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Citation	Graham, Sean M. 2016. Hemin Decreases Pro-Inflammatory Th17 Cells and Cytokines in an Adoptive Transfer Model of Colitis. Master's thesis, Harvard Extension School.
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Hemin Decreases Pro-Inflammatory Th17 Cells and Cytokines in an Adoptive Transfer
Model of Colitis

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A Thesis in the Field of Biology
for the Degree of Master of Liberal Arts in Extension Studies

Harvard University

November 2016

Abstract

The goal of this research was to reduce disease in an adoptive transfer model of colitis using an immunomodulatory therapy, hemin, while correlating this response to changes in cytokines and effector T-cell differentiation. Inflammatory bowel disease (IBD) is a consequence of immune system dysregulation, mediated by the microbiomes composition (Gálvez, 2014). IBD is most prevalent and rising in developed parts of the world such as the United States where 1.5 million people and Europe where 2.2 million people are affected (Ananthakrishnan, 2015). Research for IBD in recent years has significantly advanced our knowledge of the complex pathways and interactions involved, however a complete and long lasting treatment remains unidentified. The rate-limiting enzyme HO-1 has been found to increase pools of regulatory T-cells (T-regs), while decreasing pools of pro-inflammatory Th17 cells in a chemically induced model of colitis. Here, the in-life phase which focused on body weight and video endoscopy support other primary outcomes from multiplex immunoassays and fluorescence activated cell sorting (FACS) from blood, colon, spleen, and mesenteric lymph node samples in an adoptive transfer model of colitis in mice. Pro-inflammatory cytokines IFN- γ and TNF- α and CD4⁺ T-cells in the blood at study conclusion were found to be significantly decreased in the hemin group, as were CD4⁺CD39⁺ T-cells in the blood from days 28-56. Further, in life data suggests a positive treatment effect showing a trend with higher body weights and lower endoscopy scores. Together these data demonstrate that hemin acts in an anti-inflammatory manner by modulating pathogenic T-cell proliferation and production of key pro-inflammatory cytokines.

Acknowledgments

I would not have been able to complete this work were it not for many people supporting my goals. First, thank you to Dr. Stephen Sonis and Dr. Gregory Lyng, my thesis directors whom without, this work would not have been possible. Your encouragement, advice, support, and generosity throughout my thesis project and obtaining my Master's degree has been invaluable and greatly enhanced the quality of my experience and education at Harvard Extension School and for that I am extremely appreciative. Biomodels has been an incredible resource not only for the advanced equipment and animal models, but for the intelligent scientists and technicians who are always willing to help. As such, I would like to give a special thank you to Dr. Dominic Beal, Dr. Paulette Wright-Andreotta and the entire staff at Biomodels for all of your advice and help throughout this project.

Thank you to Dr. James Morris my thesis advisor who has been very helpful from start to finish and I appreciate the time taken to ensure my projects completion.

Finally, but of course by no means least, thank you to Ashlee who has supported me throughout this now 3-year journey with unwavering confidence and patience. I would not have been able to do this without you.

Table of Contents

Acknowledgments.....	iv
List of Tables.....	vii
List of Figures/Graphs.....	viii
I. Introduction.....	1
Inflammatory Bowel Disease Background.....	1
Gut Microbiome and T-Cells.....	4
Effector T-Cell Differentiation and Cytokines.....	6
T-Regs and Th17 Cells in Inflammatory Bowel Disease.....	9
Animal Models of Inflammatory Bowel Disease.....	11
Current Inflammatory Bowel Disease Treatments.....	12
Heme Oxygenase-1 as a Treatment Target.....	13
Research Aims, Goals, and Hypothesis.....	14
II. Materials and Methods.....	16
Adoptive Transfer Model of Colitis.....	16
FACS Analysis.....	18
Multiplex Magnetic Bead Immunoassay.....	18
Video Endoscopy.....	19
Histopathology.....	20
Heme Oxygenase-1 Activity Assay.....	20
Statistical Analysis.....	21

III. Results.....	22
In-Life Phase.....	22
Weight Change, Survival, and Diarrhea/Blood.....	22
FACS Analysis.....	23
Endoscopy Score.....	23
Post-Life Phase.....	24
Tissue Cytokine Levels.....	24
Histopathology.....	25
Heme Oxygenase-1 Activity Assay.....	26
IV. Discussion.....	27
Significance of Results.....	27
Study Limitations.....	31
Future Research Directions.....	33
Conclusions.....	34
Appendix.....	35
References.....	75

List of Tables

Table 1. In-Life Study Design.....	35
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List of Figures

Figure 1. Insult to gut results in loss of bacterial balance and a dysregulated immune response.....	36
Figure 2. T-Cell Lineage.....	36
Figure 3. Complex relationship between T-reg and Th17 balance.....	37
Figure 4. CD62L+CD44- and CD45+CD25- T-cell sort express CD45RB+	38
Figure 5. Degradation of heme by HO-1 leads to anti-inflammatory byproducts such as biliverdin.....	39
Figure 6. Representative Blood FACS Histogram measuring CD4+ T-cell counts.....	40
Figure 7. Representative Mesenteric Lymph Node FACS analysis	41
Figure 8. Representative Spleen FACS analysis.....	42
Figure 9. FACS Gating strategy.....	42
Figure 10. Day 28 Histology.....	43
Figure 11. Day 56 Histology.....	44
Figure 12. Colon Histology Scoring Guide.....	45
Figure 13. Weight changes represented by Area Under the Curve.....	46
Figure 14. Mean Group Weight Change.....	47
Figure 15. Individual Weight Change.....	48
Figure 16. Mean Group Colon Length/Weight.....	49
Figure 17. Mean blood CD4+ T-cell percentages.....	50
Figure 18. Mean blood CD4+CD39+ T-cell percentages.....	51
Figure 19. Mean Tissue CD4+ T-cell percentages.....	52
Figure 20. Mean Tissue CD4+ CD39+ T-cell percentages.....	53

Figure 21. Mean Video Endoscopy Scores.....	54
Figure 22. Video Endoscopy Scoring Scale.....	55
Figure 23. Day 14 and Day 21 Representative Endoscopy Images	56
Figure 24. Day 28 and Day 35 Representative Endoscopy Images.....	57
Figure 25. Day 42 and Day 49 Representative Endoscopy Images	58
Figure 26. Day 56 Representative Endoscopy Images	59
Figure 27. Day 28 Plasma Multiplex Graph Part 1	60
Figure 28. Day 28 Plasma Multiplex Graph Part 2.....	61
Figure 29. Day 35 Plasma Multiplex Graph Part 1	62
Figure 30. Day 35 Plasma Multiplex Graph Part 2.....	63
Figure 31. Day 49 Plasma Multiplex Graph Part 1	64
Figure 32. Day 49 Plasma Multiplex Graph Part 2.....	65
Figure 33. Day 56 Plasma Multiplex Graph Part 1	66
Figure 34. Day 56 Plasma Multiplex Graph Part 2.....	67
Figure 35. Day 56 Mesenteric Lymph Node Multiplex Graph Part 1.....	68
Figure 36. Day 56 Mesenteric Lymph Node Multiplex Graph Part 2.....	69
Figure 37. Day 56 Spleen Multiplex Graph Part 1	70
Figure 38. Day 56 Spleen Multiplex Graph Part 2.....	71
Figure 39. Day 56 Colon Multiplex Graph Part 1	72
Figure 40. Day 56 Colon Multiplex Graph Part 2.....	73
Figure 41. Representative Necropsy Photos.....	74

Chapter I

Introduction

Inflammatory Bowel Disease Background

Crohn's disease (CD) and ulcerative colitis (UC) are diseases where chronic inflammation of the epithelial lining within the intestines results from a dysregulated immune system. Together, these ailments are classified as inflammatory bowel disease (IBD). Although there has been significant progress treating IBD, many studies have shown that the incidence and prevalence of IBD has been on the rise over the past half century in developed continents such as North America and Europe. Annual incidences of UC in North America and Europe were 24.3 and 19.2 (12.7 and 20.2 for CD) per 100,000 persons-years, respectively, whereas in Asia and the Middle East were only 6.3 (5.0 for CD) per 100,000 persons-years. (Molodecky et al., 2012). 1.5 million Americans and 2.2 million Europeans are affected, and meta-analysis has shown time trend incidence increases in these continents (Ananthakrishnan, 2015). The increased incidence of IBD and the differences between populations are likely due to a variety of factors. Since IBD is an autoimmune regulated disease, data support that responses can be affected by factors such as commensal bacteria, genetic predisposition, immune response, and environmental conditions (FIOCCCHI, 2005). Furthermore, disease is perpetuated by factors produced after the initiation of disease such as oxidative stress from reactive oxygen species which damage tissue health (Rezaie, Parker, & Abdollahi,

2007). Supporting this, genetically modified mice lacking antioxidant enzymes develop spontaneous colitis (Esworthy, Kim, Doroshow, Leto, & Chu, 2013). Patients with IBD suffer from symptoms such as weight loss, pain, diarrhea, and rectal bleeding. These symptoms can often be severe and cause hospitalization during bouts of IBD (Gálvez, 2014,). The standard of care is currently up for discussion, as the emergence of immunomodulatory therapies like infliximab are becoming increasingly used over traditional therapies like 5-aminosalicylic acid or corticosteroids (Travis, 2006, Lee, Sanderson, & Irving, 2012). Typically, when treating IBD, the first goal is to limit the damage during inflammatory bouts by using 5-aminosalicylic acid and/or corticosteroids however, steroids can lead to a dependency and difficulty remaining in remission. To combat this, patients can begin treatments with immunosuppressive therapies azathioprine and methotrexate but each has several limiting factors such as a significant increase in lymphoma in azathioprine patients and hepatotoxicity in Methotrexate patients (Engel & Neurath, 2010). New biologic therapies have targeted cytokines known to be pathogenic in IBD, such the anti-TNF α monoclonal antibody Infliximab. The efficacies of these treatments are under debate and could have diminished effects due to anti-Infliximab antibodies (Nanda, Cheifetz, & Moss, 2012). Additionally, the cost of IBD in the United States per patient is over \$18,000 per year, with anti-TNF α therapies costing up to \$4,000 per infusion compared to 5-aminosalicylic acid compounds like sulfasalazine, which cost \$50 per month (Park & Bass, 2011). The newest approved therapies are integrin inhibitors like vedolizumab, which block T-cell transportation to the gut (Feagan et al., 2013).

The main cell type involved in the autoimmune response is T-cells. The potential for T-cells to differentiate into a specific lineage: Th1, Th2, Th17, or T-regs plays a key role in the development of the autoimmune response in IBD and other autoimmune diseases (Cheng et al., 2009, Dieleman et al., 1998, Luger et al., 2008). Therefore, it is critical to fully understand and characterize T-cell distribution, differentiation, and activation pathways when researching this complex disease. Immune system dysregulation is a hallmark of IBD, the balance of T-cell subsets plays a critical role in establishing gut homeostasis but can be disrupted by a combination of genetic, environmental, and host microbiome factors. Furthermore, the balance between and effect of each subset is largely mediated by cytokines (Gálvez, 2014, Kucharzik et al., 2006). More recently, there has been a strong interest in understanding the role of genetic factors, such as mutations to the IL-10 receptor being associated with early onset CD (Colombel, 2014). Findings such as these highlights the importance of treating IBD patients individually, as there may be very specific differences leading to different treatment avenues. Combination therapy based on individual patient immune responses, microbiota, and genetic factors are likely the next approach in developing new IBD treatments. Identifying and fully elucidating the pathways involved in IBD remains the most important treatment variable. As such, research that focuses on further unravelling these pathways and how they interact with each other is highly needed.

Gut Microbiome and T-Cells

The intestinal lining is the first barrier in host defense, limiting pathogenic responses by functioning as a semi-permeable wall. When this barrier is damaged by local inflammation, it becomes more permeable, allowing passage of bacteria, electrolytes, antigens, and other macromolecules, which attribute to the pathogenesis of IBD (Berkes, 2003, Liu, Li, & Neu, 2005). While paracellular and transcellular passage are normal functions of the epithelium, these are increased during the inflammatory response and coupled with passage through lesions and can lead to the development of disease (Lewis & McKay, 2009, Lewis et al., 2010). This insult to the tissue coincides with changes in the way the host microbiota is recognized by T-cells, ultimately leading to dysregulation of the immune system signified by increased T-cell activation and differentiation of pathogenic T-cells (Figure 1) (Gálvez, 2014). The immune response and dysregulation in IBD is in large part mediated by effector T-cells Th1, Th2, Th17, and T-regs. Th1 and Th2 cells were initially the focus of IBD research, however since the discovery of Th17 cells and T-regs a large importance has been placed on elucidating their roles in autoimmune disorders. The balance between Th17 cells and T-regs, discussed in detail later, has been established as a chief regulator of autoimmune disease (Abdulahad, Boots, & Kallenberg, 2010, Berger, 2000).

The bacterial flora composition in the gut is of particular importance in the context of IBD due to its ability to either stabilize or initiate an immune response by affecting T-cell responses. Decreased complexity of the gut microbiome has been linked to increased disease; stress, diet, genetics, drugs, and other factors can lead to losing the

stability of gut homeostasis and allow the development of chronic inflammation (Kostic, Xavier, & Gevers, 2014). Demonstrating how vital the microbiome is to disease, studies conducted in germ free animals show that disease severity in the DSS model is repressed (KITAJIMA, MORIMOTO, SAGARA, SHIMIZU, & IKEDA, 2001). Indeed, bacteria such as *Escherichia coli* and the genus *Fusobacterium* have been found at increased levels in IBD patients (Kostic, Xavier, & Gevers, 2014). While the microbiome as a whole is needed and partially responsible for disease development, there are specific bacteria that can be helpful. For instance, *Clostridium*, a common intestinal bacterium can be anti-inflammatory by inducing T-reg proliferation (Chiba & Seno, 2011). Findings such as these indicate that researching responses to specific bacteria could potentially lead to new therapeutic targets. The microbiome, having this bimodal functionality to stimulate and inhibit disease highlights the importance and complexity of the microbiome when considering the pathogenesis of IBD by T-cells. Also playing a role in immune system sensitivity to the microbiome are genetics. NOD2, responsible for activation of transcription factor NF- κ B in monocytes is associated with CD when mutated (Hugot et al., 2001). While NOD2 can have different variations of mutations, pathogenic mutations lead to over activation of NOD2/ NF- κ B leading to increased monocyte proliferation and microbial recognition (Hugot et al., 2001). Advancements increasing our understanding of the relationship between host microbiome and genetics are important due to the complexity of IBD's pathogenesis. With these new findings, it appears that treatments in the future will need to consider the patients' specific immune response, bacterial flora, and genetic background.

Effector T-Cell Differentiation and Cytokines

T-cell precursors go through negative selection in the thymus, which forces cells that have self-reactivity to undergo apoptosis, thus preventing the adaptive immune response from attacking its own healthy cells. Self-tolerant T-cells then become effector T-cells, derived from these naïve precursors, and the conditions in the cell, the microbiome, and genetics can drive the proliferation of specific T-cell subsets (Gálvez, 2014, Josefowicz, Lu, & Rudensky, 2012). While there is a range of phenotypically distinct effector T-cells, they can be identified based on the expression of particular cell surface molecules; these markers not only allow for the identification of subsets, but also directly determine effector functionality. The most basic subsets of T-cells are the CD4⁺ and CD8⁺ lineages. The CD8⁺ lineage T-cells become cytotoxic killers, whereas the CD4⁺ lineage have the potential to become Th1, Th2, Th17, or T-reg (Germain, 2002, Harty, Tvinnereim, & White, 2000) (Figure 2.). The differentiation into Th1, Th2, Th17, or T-reg lineages is determined by interactions between the naïve T-cell, antigen presenting cells, and the cytokine milieu. Cytokines can either be produced by the T-cell or surrounding cells and effect the immune response. One of the ways cytokines affects the immune response is by driving T-cell differentiation toward a specific T-cell phenotype. Certain cytokines are strongly associated with each subset, for example, TGF- β is associated with more Th17 differentiation and IFN- γ both stimulates differentiation into and is produced by Th1 T-cells (Zhu, Yamane, & Paul, 2010). Improperly balanced effector T-cells Th1/Th2/Th17 and T-reg patterns are associated with numerous disease states (Cheng et al., 2009, Dieleman et al., 1998, Luger et al., 2008). Cytokines are

therefore very important in maintaining the balance between these cell lineages which is vital for sustaining a healthy immune system.

Th1 cells are typically associated with a pro-inflammatory cytokine profile, of which the quintessential cytokine is interferon- γ (INF- γ). T-cell precursors are stimulated to differentiate into Th1 cells by INF- γ , Th1-cells then also produce INF- γ , generating a feedback loop (Zhu, Yamane, & Paul, 2010). This feedback loop is associated with unregulated autoimmune responses and tissue damage and is a treatment target due to its ability to potentiate disease (Berger, 2000, Resta–Lenert & Barrett, 2006, Zhu, Yamane, & Paul, 2010). Indeed, inflammation in CD is mediated Th1 and Th17 cells again signifying the importance of INF- γ in IBD (Fuss, 2008, Zhang, Fuss, Yang, & Strober, 2013). Research however also indicates that disease may be shunted towards a Th1 or Th17 response, finding that INF- γ can promote Th17 development and stop Th1 development through interactions with antigen presenting cells (Kryczek et al., 2008). By contrast, Th2 cells are involved in anti-helminthic and allergic responses, while also being antagonistic to the Th1 response in allergic responses (Rotmagnani, 1998). Th2 responses are associated with the cytokines IL-4 and IL-13, which have been associated with the pathogenesis of UC (Fuss, 2008). IL-4 stimulates naïve T-cell differentiation into Th2 cells, which then produce both IL-4 and IL-13, treatments targeting these have not been successful (Fuss, 2008, Zhu, Yamane, & Paul, 2010).

IL-12 and IL-23 are dimers, which both consist of one p40 subunit. IL-12 has been implicated in Th1 type inflammatory responses, while IL-23 is important in the activation of innate immunity such as monocyte activation (Langrish et al., 2004). Past research had indicated that treatment with anti-p40 monoclonal antibodies worked by

repressing Th1 T-cells, but it has since been discovered that anti-p40 is also responsible for repressing Th17 responses through an IL-23 blockade (Luger et al., 2008). Th17 cells, like Th1 cells have been described in many autoimmune diseases, are induced by IL-6 and IL-23 and are most well known for producing the pro inflammatory cytokine IL-17 (Weaver, Harrington, Mangan, Gavrieli, & Murphy, 2006). Th17 cells also produce the transcription factor ROR- γ which further induces the generation of Th17 cells, perpetuating inflammation. When ROR- γ is missing, disease dwindles and the affected tissue does not have large infiltrates of pathogenic Th17 cells (Ziegler & Buckner, 2009). T-regs on the other hand are the primary suppressive cells involved in the reduction of pro-inflammatory responses, which are characterized by the expression of transcription factor Foxp3 and production of the immunosuppressive cytokine IL-10 (Maynard et al., 2007). The presence of Foxp3 allows T-regs to act as potent immune suppressors, and when missing from the immune system a homeostatic environment cannot be established resulting in autoimmune disorders (Josefowicz, Lu, & Rudensky, 2012). The balance between the expression levels of these two transcription factors ROR- γ and Foxp3, observed in healthy individuals, is the result of a complex pathway with multiple routes of induction and repression of Th17 cells and T-regs; when perturbation of this delicate balance is thought to be a major factor in the pathogenesis of autoimmune disorders (Ziegler & Buckner, 2009).

T-Regs and Th17 Cells in Inflammatory Bowel Disease

In recent years, researchers have identified Th17 cells as a powerful driving force in the inflammatory cascade causing irregular autoimmune function. As such, studies in animal models of colitis have shown that disease can be attenuated by either blocking differentiation of Th17 cells, or blocking IL-17, the main inflammatory cytokine produced by Th17 cells (Gálvez, 2014). Additionally, blocking the Th17 associated transcription factor ROR- γ also results in diminished disease (Zhang, Fuss, Yang, & Strober, 2013). Further research has shown that a portion of Th17 cells can shift their function towards a Th1 like phenotype that are necessary for the pathogenesis of IBD (Harbour, Maynard, Zindl, Schoeb, & Weaver, 2015).

T-regs play a critical role suppressing autoimmune responses including the inflammation caused by these pathogenic Th17 cells. T-regs express the co-stimulatory molecule CTLA-4, which is critical for T-reg mediated suppression (Josefowicz, Lu, & Rudensky, 2012). IL-2 was initially a target for IBD treatment, however it became clear that it was not imperative to have IL-2 present in order to perpetuate disease (Schimpl et al., 2002). When CTLA-4 is blocked however, T-regs lose their suppressive effectiveness allowing autoimmune disease to arise (Sakaguchi, Wing, Onishi, Prieto-Martin, & Yamaguchi, 2009). This vital suppression function allowing gut homeostasis is supported by the findings that when CD4⁺CD25⁺ cells, markers associated with T-regs, are adoptively transferred into diseased SCID CD4⁺CD25⁻ mice, disease is fully reversed (Liu, Hu, Xu, & Liew, 2003). The balance between pathogenic T-cells and non-pathogenic T-regs represents a weak spot of the immune response, viruses have been

found to target this by causing T-regs to undergo apoptosis resulting in a dysregulated autoimmune response and chronic infections, using a correlate human respiratory viral infection mouse model using sendai viral infections (Bharat et al., 2010).

Research has shown that the relationship between T-regs and Th17 cells is very complex (Figure 3). Under inflammatory autoimmune conditions where IL-6 is being secreted, such as found in rheumatoid arthritis, it has been shown that T-regs can be converted into the Th17 phenotype, further driving the inflammatory response (Komatsu et al., 2013). Clinical data from IBD patients supports this discovery; patients had increased CD4⁺ T-cells expressing both IL-17 and Foxp3, suggesting phenotype conversion from T-reg to Th17 (Ueno et al., 2013). Conversely, Th17 cells have also been shown to stop secreting pro-inflammatory cytokine IFN- γ and promote generation of IL-22, which decreases epithelial tissue damage by promoting microbial resistance responsible for disease abrogation (Gálvez, 2014). While both cells have been found to have functions opposite of their normal state, they can also inhibit one another. When the receptor for immunosuppressive cytokine IL-10 loses function in patients, the Th17 levels increase dramatically (Shouval et al., 2015). The regulation of T-reg proliferation is also controlled by epigenetic factors such as the recently described methyltransferase Uhrfl. Silencing of Uhrfl results in spontaneous colitis due to an increased load of Th17 cells and loss of function of T-regs. (Obata et al., 2014). Finally, corroborating these results, further research has shown that Uhrfl loss results in a TNF α based inflammatory cascade leading to the loss of immune homeostasis in the gut (Marjoram et al., 2015). Together these recent findings represent an important connection between T-reg function and development of IBD due to the actions of Th17 cells.

Animal Models of Inflammatory Bowel Disease

The complexity of IBD has been difficult to unravel. Until recently, the only options for inducing colitis in an experimental setting were chemical, such as dextran sodium sulfate (DSS), tri-nitrobenzene sulfonic acid (TNBS), and oxazolone (Wirtz, Neufert, Weigmann, & Neurath, 2007). TNBS predominantly confers a Th1 response, oxazolone a Th2 response, and DSS a mixed Th1/Th2 response (Wirtz, Neufert, Weigmann, & Neurath, 2007, Dieleman et al., 1998). While these models have been useful in the research of IBD, the field has been focusing further on the microbiome and T-cells. The development of adoptive transfer mediated models of colitis allows the investigation of the pathogenesis pathways in a setting where T-reg's are missing and naive T-cells are undergoing differentiation into their effector subsets (Kanai et al., 2006). With such importance having been found in the balance between Th17 cells and T-reg's, as discussed in the previous section, being able to evaluate a treatment's effect on T-cell proliferation is of high interest. The CD45⁺CD25⁻ T-cell adoptive transfer model researchers are now able evaluate the differentiation and homing of T-cells beginning from the naïve state, a truly unique aspect to the model made possible by isolating naïve CD45^{RB} HIGH T-cells (Ostanin et al., 2008). A similar naive T-cell sort and transfer for CD62L⁺CD44⁻, used in this research, also induces disease by isolating the CD45^{RB} HIGH T-cells (Figure 4). Some advantages of this model include being able to study the earliest stages of disease while focusing T-cell differentiation and proliferation (Ostanin et al., 2008). The details of this model will be discussed further in Chapter II.

Current Inflammatory Bowel Disease Treatments

The most effective treatments have aimed to alter the T-cell response through cytokine inhibition/depletion efforts such as anti-p40 and anti-TNF α . Anti-p40 which depletes IL-12 and IL-23 results in the blunting of Th1 and Th17 responses by suppressing a key Th17 cytokine IL-17A, has shown efficacy in early testing (Kim et al., 2012). The discovery of treatments targeting the highly pro-inflammatory cytokine TNF α with antagonistic monoclonal antibodies resulted in the most currently viable treatment option (Rutgeerts, van Assche, & Vermeire, 2004). The ability of TNF α antagonists to trigger apoptosis in monocytes and lymphocytes has led some to question the susceptibility of anti-TNF α patients to infectious disease however, little evidence has been found to support this concern (Targownik & Bernstein, 2013). Instead, the primary worry of anti-TNF α therapy is patient variability, there is a significant number of patients that do not have either a short or long term response to treatment. In clinical trials, up to 40% of patients do not respond to anti-TNF α therapy and up to 46% of responders lose response within one year of treatment (Ben-Horin, Kopylov, & Chowers, 2014). When drugs such as Infliximab, a common anti-TNF α antibody and IBD treatment, or other standards of care such as antibiotics and steroids do not work, the last resort for patients is to undergo colectomy in order to surgically remove diseased portions of the intestines (Sandborn et al., 2009). Antibiotics have been found to have moderate but varied success in treatment (Khan et al., 2011). Steroids are also used in IBD patients, but long term steroid treatment is not ideal due to its limited response window (Hanauer, 2002). Even with great progression in recent years, the large number of non-responders found among

the best treatment option, anti-TNF α , indicate alternate routes in the pathways leading towards IBD related inflammation.

As discussed in previous sections, the balance between Th17 and T-regs has advanced to the forefront of IBD research. As recently as 2016, it has been shown that mTOR inhibition can function as an IBD treatment due to its ability to restore this critical balance in a DSS model of colitis (Hu et al., 2016). Another possible route to treat the Th17/T-reg balance is by modulating the microbiome; short chain fatty acid producing bacteria, such as the *Clostridia* species are implicated in the proliferation of T-regs is significantly reduced in IBD patient fecal samples (Omenetti & Pizarro, 2015). Considering how many treatments show efficacy, it is clear that there are many pathways leading to the complexity and downfall in identifying a complete cure. Instead, it appears that the long term treatment of IBD will require an approach that involves multiple mechanisms of action.

Heme Oxygenase-1 as a Treatment Target

Heme oxygenase-1(HO-1) has been identified recently as a new treatment target in IBD (Onyiah et al., 2013). HO-1 is an enzyme which degrades heme into the antioxidants biliverdin, iron, and carbon monoxide (CO) (Naito, Takagi, & Yoshikawa, 2004). Biliverdin is possibly the most important product of this reaction, it leads to reactive oxygen species scavenging, anti-complement, and inhibition of neutrophil and CD8+ T-cell influx (Naito, Takagi, & Yoshikawa, 2004) (Figure 5). Oxidative stress plays a major role in the pathogenesis of colitis, and the byproducts generated by HO-1

makes it a natural source of induced antioxidants, which have been found to provide protection against IBD (Oz, Chen, McClain, & de Villiers, 2005). Additionally, oxidative stress has been found to result in dysfunctional DNA methyltransferases, which normally act as anti-inflammatory molecular switches by repressing TNF α production (Low, 2013, Marjoram et al., 2015). In relation to recent work in T-regs and Th17 cell balance, in a recent DSS model based study, HO-1 induced by hemin was found to not only result in a depletion of Th17 cells but also an increase in the pool of T-regs compared to untreated controls (Zhang et al., 2014).

Research Aims, Goals, and Hypothesis

The primary research goal of this thesis was to assess the efficacy of heme oxygenase-1 (HO-1) on altering T-cell differentiation and ratios in an adoptive transfer model of CD4⁺ T-cell colitis in mice. Based on previous literature, it was hypothesized that hemin would have a profound effect in diminishing disease in an adoptive transfer model of colitis, measured by weight loss and video endoscopy score. Moreover, this effect would correlate with a reduction in pro-inflammatory cytokines and more specifically a skew away from a Th17 response. To assess this hypothesis, two specific aims work together to answer the primary objective. Details of the study design can be found in Table 1.

Primary objective: Show disease reduction in an in vivo model of colitis and correlate this change with T-cell profile modulation and cytokine differences.

Specific Aim 1: Show disease reduction by treatment with hemin using compilation of measurements in a standard model of IBD.

Methods: Track weight change, evaluate disease via video endoscopy, monitor blood/diarrhea presence around the rectum, and colon weight/length change.

Expected results: There will be a reduction in disease represented by reduction in weight loss, endoscopy score, blood/diarrhea presence, and colon weight/length ratio.

Specific Aim 2: Show a reduction in proinflammatory T-cells and cytokines using FACS and immunoassays in a standard model of IBD.

Methods: Quantitate CD4+, CD4+CD25+, and CD4+CD39+ T-cells using FACS analysis on the blood, spleen, and mesenteric lymph nodes. Additionally, the blood, spleen, mesenteric lymph nodes, and colon will be analyzed using immunoassays for IFN- γ , IL-1b, IL-4, IL-6, IL-22, IL-10, IL-23, IL-12, IL-13, IL-17A, TNF- α , and TGF- β 1.

Expected Results: There will be a reduction in CD4+ and CD4+CD39+ T-cells in the hemin treatment group as well as an increase in CD4+CD25+ T-cells. Furthermore, proinflammatory cytokines will be reduced in blood and tissue samples.

Chapter II

Materials and Methods

Animals

A total of 16 male C57BL/6-*Rag2^{tm1Cgn}*/J and 10 male C57BL/6 mice from Taconic Biosciences (Germantown, NY) were used in this study. RAG2 (recombination activating gene) is transcribed and translated into a protein primarily responsible for the rearrangement of genes encoding T and B cells receptors; without proper functional rearrangement the cells are signaled for apoptosis (Shinkai, 1992). The donor C57BL/6 mice provide CD4⁺ T-cells that are able to be stimulated to differentiate and proliferate in the RAG2 knockout mice. The KO mice were bred on a C57BL/6 background and are MHC matched. These KO mice were used for the disease phase portion of the study, comprising two groups of 8 mice each.

Adoptive Transfer Model of Colitis

To obtain naive T-cells to induce colitis with, the spleens were harvested from the 10 C57BL/6 donor mice and prepared for magnetic bead separation. First, the spleens were placed in ice cold PBS upon excision. Next, the spleens were homogenized using a gentleMACS dissociator (Miltenyi, San Diego, CA) in order to create a single cell suspension. Finally, the suspension of splenocytes was sorted using a MACS column

(Miltenyi, San Diego, CA) to isolate naïve T-cells, defined by the cell surface molecules CD4⁺CD62L⁺CD44⁻. Once sorted, the cells were counted and resuspended in PBS such that each recipient RAG2^{-/-} mouse received a 200µl intraperitoneal dose containing 0.5×10^6 naïve CD4⁺ T-cells. When injected, the naïve CD4⁺ T-cells are stimulated by interactions with the microbiome to proliferate and differentiate (Feng, Wang, Schoeb, Elson, & Cong, 2010).

Outward signs of inflammation begin at 5-8 weeks after transfer, and can be detected by weight loss, video endoscopy, and the presence of blood and/or diarrhea near the rectum. After induction (Day 0), weight change and incidence of blood/diarrhea was recorded daily, and endoscopy performed once a week beginning day 14. On day 0 animals began treatment with either PBS or hemin (50µg/kg) intraperitoneally twice a week until study conclusion. Additionally, at the time points indicated in Table 1, retro-orbital blood collections were performed to count CD4⁺, CD25⁺, and CD39⁺ T-cells using fluorescence activated cell sorting (FACS) and IFN- γ , IL-1b, IL-4, IL-6, IL-22, IL-10, IL-23, IL-12, IL-13, IL-17A, TNF- α , and TGF- β 1 immunoassays. Also, mesenteric lymph nodes and spleen were collected at sacrifice and analyzed using the same methods as the blood. Finally, on day 28 and 56 animals were sacrificed for organ collection, specifically the colon, spleen, mesenteric lymph nodes, and blood. When the colon was excised it was measured, weighed, contents flushed, and trimmed to a length of 5 cm from the rectum where disease was assessed by endoscopy. From the 5 cm section a 1 cm middle piece was taken for multiplex ELISA cytokine immunoassays and HO-1 activity assay. The remaining two 2 cm sections were used for histopathological analysis, discussed further in the following sections.

FACS Analysis

Whole blood samples were prepared in K2EDTA tubes each week beginning day 14, except for day 49 due to equipment restrictions. Also, mesenteric lymph nodes and spleen were collected at sacrifice. Tissue and blood samples were prepared for analysis by FACS for the expression of cell surface markers CD4, CD8, CD25, and CD39, as follows: solid tissues were dissociated in ice cold FACS buffer (5% BSA, 5mmol K3EDTA in 1x PBS) using the gentleMACS system to yield a single cell suspension. Blood and spleen were RBC lysed (BD Pharmlyse, Franklin Lakes, NJ) 1.0×10^6 cells were aliquoted and stained with fluorescent antibodies specific for CD4, CD8, CD25, and CD39, and run through a miltenyi MACSquant flow cytometer (Miltenyi, San Diego, CA). Data was analyzed using FlowJo software and representative FACS plots are seen in Figures 6 to 8. CD39⁺ T-cells were calculated as a percentage of CD4⁺ T-cells. The gating strategy, seen in Figure 9, was used to identify populations of CD4⁺ T-cells that are also CD39⁺ and CD25⁺.

Multiplex Magnetic Bead Immunoassay

A portion of the colon was used in multiplex cytokine panel immunoassays, to detect IFN- γ , IL-1b, IL-4, IL-6, IL-22, IL-10, IL-23, IL-12, IL-13, IL-17A, TNF- α , and TGF- β 1 (EMD Millipore Corporation). Colon samples were prepared by low to medium homogenization for 30 seconds in PBS while on ice to prevent degradation. The protocol supplied by the vendor was as followed; briefly, the 96 well immunoassays were loaded

with appropriate standards and controls provided by the Milliplex MAP Kit, magnetic beads to mark the specific cytokines of interest, and homogenized sample. Following the 2 hour incubation period, the plate was read using a Luminex200 to obtain the median fluorescent intensity (MFI) which was then used to calculate cytokine concentrations using the curve generated by the standards for each analyte. Day 28 tissues were not used in this analysis due to limited reagents.

Video Endoscopy

Beginning on Day 14 and then once weekly throughout the study duration, colitis severity was assessed in all animals using high definition video endoscopy where images were taken and colitis severity scored by a blinded observer according to the scoring scale seen in Figure 22 (Hamilton et al., 2011, Lyng, Stevens, Gordon, Watkins, & Sonis, 2008). Animals were anesthetized using 2.5% isoflurane vapor, for each animal at each time point, the endoscope was placed into the rectum using a small amount of lubricant. A high definition camera was attached to the endoscope (Karl Storz, Germany) which was 14cm in length and rigid with a beveled tip. When the endoscope passed the rectum, a small amount of air was pushed into the colon through the endoscope biopsy channel to inflate, allowing visualization of the tissue. The endoscope was then carefully moved down the length of the colon pushing any stool, mucous, or blood towards the proximal end. While removing the endoscope the tissue was clear of debris and inflated to visualize and give the appropriate score by a blinded observer. Following the procedure all animals' recovery from anesthesia was monitored. Video endoscopy can be a

powerful measure of colitis severity, in the adoptive transfer model used here the disease is mild-moderate compared to the more severe chemically induced models like DSS and TNBS.

Histopathology

Colon samples obtained from sacrificed animals were sectioned for histopathology. Of the 6-8cm colon tissue, two 2 cm sections were collected in 10% neutral buffered formalin. These sections were then stained with hematoxylin and eosin (H&E) and sectioned 8-10 times at 5-8 μ m thickness per 2cm tissue on each slide. Histology photos displaying disease progression for each group on days 28 and 56 are shown in Figures 10 and 11, selected by an unblinded observer guided by the colon histopathology scoring scale (Figure 12).

Heme Oxygenase-1 Activity Assay

A previously described enzyme activity assay for HO-1 was used, with modifications (Zhang et al., 2014). After running test samples using C57Bl/6 colon tissue, steps were taken to increase the sensitivity of the assay. In order to compensate for the small protein content and sample size, changes were made to decrease the dilution of the sample. The original protocol is as follows “The colons were homogenized in 10 mmol/liter HEPES, 32 mmol/liter sucrose, 1 mmol/liter dithiotreitol (DTT), 0.1 mmol/liter EDTA, 10 μ g/ml soybean trypsin inhibitor, 10 μ g/ml leupeptin, and 2 μ g/ml

aprotinin and centrifuged at $18,000 \times g$ for 30 min at 4 °C. The supernatant was used to measure HO activity. The reaction mixture, consisting of 200 μ l of sample homogenate, 100 μ l of normal liver cytosol (source of biliverdin reductase), 20 μ mol/liter hemin, and 0.8 mmol/liter NADPH, was incubated at 37 °C for 1 h. The optical density (OD) was measured between 464 and 530 nm (extinction coefficient, 40 mmol/liter/cm for bilirubin) to assess bilirubin production once the reaction was terminated by putting samples in an ice bath. Values were expressed as pmol of bilirubin formed per hour per milligram protein” (Zhang et al., 2014). Here, the reaction mixture consisted of 100 μ l of sample homogenate and 50 μ l of normal liver cytosol. The OD was measured at 450 nm and 540 nm which were the closest filters available.

Statistical Analysis

All quantitative values are expressed as the average of the group \pm standard error of the mean (SEM). All statistics except for endoscopy scores were analyzed and graphed using a one-tailed t-test between Vehicle and Treatment groups in Prism (Graphpad). Endoscopy scores were tested using Mann-Whitney U tests. P-values were considered significant if $p \leq 0.05$. Values falling below the limit of detection in the cytokine panels were expressed as 50% of the lowest standard. Values above the limit of detection in the cytokine panels were expressed as the highest standard detected. Animal number 4 had extremely high outliers found in the immunoassay data, as such the data was not included in the analysis.

Chapter III

Results

In-Life Phase

Weight Change, Survival, and Diarrhea/Blood

Weights were obtained daily throughout the course of the study and percent change was calculated each day relative to the baseline weight set on day 0. Over the duration of the study, no animals were sacrificed due to weight loss or morbidity however, one hemin treated animal was found dead on day 52. Disease in the adoptive transfer model takes roughly 25 to 40 days to initiate weight loss. Here, weight loss was observed in this window, beginning day 28. Weight change through day 28 and 56 expressed as area under the curve (Figure 13) shows nearly identical weights on day 28 and higher weights for the hemin group on day 56. Overall, the hemin group was trending with less weight loss than the vehicle group, however statistical significance was not reached until the final two days of the study (Days 55 and 56). Mean daily group weights (Figure 14) show similar weight change patterns, however the hemin group tended to have less weight loss especially towards the end of the study. Individual animal weight change (Figure 15) shows distribution within groups, the hemin group was mostly higher than the vehicle group other than one animal. Diarrhea and blood checks were performed daily, however there were no observed bouts of either. Colon weight and length was

measured when harvested and found no significant difference between the two groups (Figure 16). Additionally, although no toxic side effects were noticed, upon sacrifice the hemin treated animals had deposits of hemin in the abdomen (Figure 41).

FACS Analysis

Labels for CD4, CD8, CD25, and CD39 were used to determine T-cell levels. No distinct CD25+HIGH (T-regs) or CD8+ (Cytotoxic T-Cell) populations were identified indicating an accurate cell sort for naïve CD4+ T-cells. On day 56, the blood CD4+ T-cell percentage was significantly higher in the vehicle group (Figure 17). Further, on days 28, 35, 42, and 56 there were significantly increased levels of CD39+ cells, calculated as a percentage of CD4+ T-cells (Figure 18). In the mesenteric lymph nodes collected on days 28 and 56, CD4+ T-cells and CD4+CD39+ T-cells were the same for each group, however in the spleen while not significant there tended to be higher CD4+ and CD4+CD39+T-cell percentages in the vehicle group (Figures 19 and 20).

Endoscopy Score

Video endoscopic evaluation of the colon performed weekly beginning on day 14 resulted in no significant differences between groups, however the average scores in the hemin group were trending lower beginning on day 35 (Figure 21). Representative images from each week (Figure 23 to 26), show gross morphological changes between groups. These images best represent the mean group values, selected by an unblinded

observer, and a trend can be seen with more disease severity in the vehicle group particularly at the last 3 evaluations where each animal reached a colitis score of 2, whereas the hemin group has animals with colitis scores of 1.

Post-Life Phase

Tissue Cytokine Levels

Twelve cytokines were evaluated, IFN- γ , IL-1b, IL-4, IL-6, IL-22, IL-10, IL-23, IL-12, IL-13, IL-17A, TNF- α , and TGF- β 1. Values were obtained for each in the blood plasma on day 28, 35, 49, and 56 and at sacrifice on day 56 the mesenteric lymph nodes, spleen, and colon. Figures displaying the complete results can be found in the appendix (Figures 27 to 40)

On day 28 in the blood plasma, there were no significant differences between groups (Figure 27 and 28). However, the pro-inflammatory cytokines IFN- γ and TNF- α levels in the vehicle group were trending higher than the hemin group. This observation was confirmed by following findings on days 35 and 49 where the trend was still widening, and on day 56 where ultimately there was a significant difference (Figures 29 to 34). Mean IL-17A values for each week of plasma samples were below the limit of detection (LOD), but on day 35 the findings showed a significant decrease in of IL-17A in the hemin treatment group (Figure 30). Again on days 49 and 56, IL-17A values were below the LOD but still trending with higher values for the vehicle group (Figures 32 and

34). Additionally, on days 49 and 56 TGF- β 1 levels were trending with higher values in the hemin group (Figures 32 and 34).

Mesenteric lymph nodes taken at study conclusion showed significant differences with higher values in the vehicle than the hemin for cytokines IL-12, IL-23, and IL-13 (Figure 36). IL-1b, IL-4, IL-6, IL-10, and TNF- α tended to be higher in the vehicle group, while IL-22 and TGF- β 1 were higher in the hemin group (Figures 35 and 36). The spleen samples had significant values for 10 of 12 cytokines tested, only IL-4 and TGF- β 1 were not found to be significant. IL-4 appeared to be higher in the vehicle group but not significant (Figures 37 and 38). In the colon, TGF- β 1 was significantly higher in the vehicle group (Figure 40). IFN- γ , IL-1b, IL-4, IL-6, IL-23, IL-12, IL-13, IL-17A, and TNF- α were trending higher in the vehicle group but not significant (Figures 38 and 39).

Histopathology

Representative histopathology images show gross morphological changes in the epithelial and muscular layer of the colon (Figures 10 and 11). Disease progression marked by morphological changes can be seen including edema, neutrophil infiltrate, and crypt morphology loss. Figure 12 is a representative histopathology colon scoring scale with scoring descriptions.

Heme Oxygenase-1 Activity Assay

No detectable changes in optical density were observed, therefore the presence of HO-1 was not confirmed. Limitations and future research based on this is discussed in the study limitations section.

Chapter IV

Discussion

Significance of Results

Heme oxygenase-1 (HO-1) is an oxidative stress response enzyme that catalyzes the breakdown of heme into carbon monoxide, biliverdin, and iron (Figure 5) (Naito, Takagi, & Yoshikawa, 2004). HO-1 is a rate-limiting enzyme that has been identified as an autoimmune suppressor in a variety of disease models (Choi & Alam, 1996, Panahian, Yoshiura, & Maines, 2008). Recently, treatment with the HO-1 inducer hemin has been shown to have efficacy in the DSS-induced colitis model (Zhang et al., 2014). Specifically, numbers of pro-inflammatory Th17 cells were decreased and suppressor T-regs were increased which resulted in a significant treatment effect, diminishing disease (Zhang et al., 2014). Here, we saw a similar effect on T-cell modulation in an adoptive transfer model of colitis using FACS analysis and immunoassays while monitoring disease severity using weight loss and video endoscopy as primary endpoints.

Throughout the course of the 57 day study, animal body weights were obtained daily. Notably, both groups had similar weight change until separation began after day 42, with the hemin treated animals tending to show less weight loss (Figure 14). Near study conclusion on days 55 and 56 the difference in weight change reached statistical significance, where the hemin group has shown an overall gain in weight. The individual weight change graph shows the distribution of weight loss within groups, where it can be seen that the hemin group animals are trending higher than vehicle with one exception

(animal #11) (Figure 15). Importantly, weekly endoscopic evaluation revealed that the vehicle group reached a peak score of 2 for all animals on day 42 which persisted until study conclusion, while the hemin group had an average score of 1.6, 1.6, and 1.8 on days 42, 49, and 56 respectively. However, this effect did not reach statistical significance (Figure 21). Although endoscopy was not significant, a trend is seen in the representative photos, Figures 23 to 26, where the hemin treated animals have less tissue friability and/or bleeding. These findings together suggest a positive treatment trend for the hemin group. Colon weight to length ratios were calculated to determine gross physiological changes, but differences were not found between groups.

To support these in-life endpoints, beginning on day 14, a small volume of peripheral blood (100 μ L) was collected from each animal once per week; plasma was collected for subsequent immunoassays, and the cellular portion was reserved for FACS analysis. In addition to blood, mesenteric lymph nodes, spleen, and colon were harvested and homogenized for these analyses at time of sacrifice (Table 1). First, CD4⁺ T-cell populations were identified by FACS analysis, which showed a significant increase of CD4⁺ numbers in the vehicle group compared to the hemin group on day 56 in the blood. We measured for CD25 and CD39 expression within the CD4⁺ populations, finding that there were no CD25 high events (Figure 6). T-regs are characterized by high expression levels of the cell surface marker CD25 (IL-2R) (Josefowicz, Lu, & Rudensky, 2012). With the results showing no CD25 high populations, and knowing that CD25⁺ cells were removed during the initial cell sort, we can conclude there are no significant true T-reg populations (Beyer, 2006). While this initially was seen as a deviation from the findings from the DSS models, we found that CD4⁺CD39⁺ T-cells were significantly increased in

the vehicle group each week beginning day 28 and persisted until study conclusion (Figure 18). The cell surface marker CD39 has been shown to be present on both pathogenic Th17 and T-regs (Bai et al., 2014, Dwyer et al., 2010). Since there are no T-regs present as (defined here as CD4+CD25^{high}), these data indicate a significant increase in the pool of Th17 cells in the vehicle group compared to the hemin. No significant differences were found in the spleen and mesenteric lymph nodes, using FACS analysis (Figures 19 and 20).

Multiplex immunoassays were used to detect the presence of IFN- γ , IL-1b, IL-4, IL-6, IL-22, IL-10, IL-23, IL-12, IL-13, IL-17A, TNF- α , and TGF- β 1 in the peripheral blood plasma, mesenteric lymph nodes, spleen, and colon. IFN- γ and TNF- α levels were both significantly elevated in the vehicle group compared to the hemin group on day 56 in the blood. This finding suggests that hemin is suppressing the secretion and/or production of pro-inflammatory cytokines. Interestingly, IL-17A levels were found to be significantly increased on day 35 in the vehicle group, while trending similarly for the remainder of the study (the significance of this result is limited due to the mean IL-17A levels were below the limit of detection). Low levels of IL-17A suggest low overall levels of Th17 cells and/or a diminished response, however, the number of T-cells in the adoptive transfer model is much less than that of a wild type C57Bl/6 mouse; this would account for multiple target cytokines falling below the limit of detection (Figures 27 to 40). In a wild type C57Bl/6 mouse lymphocytes comprise over 60% of the cells in the blood, compared to just over 10% on day 56 in the vehicle group (Figure 17) (JAX Physiological Data Summary - C57BL/6J, 2016). Supporting this is the finding that there were significantly increased levels of IL-17A in the spleen well over the limit of

detection (Figure 38) and the level of T-cells in the spleen were close to that of a wild type C57Bl/6 mouse spleen (Figure 19) (JAX Physiological Data Summary - C57BL/6J, 2016). In the colon, TGF- β 1, levels were significantly higher in the vehicle group than the hemin group (Figure 40). This finding is interesting because literature references indicate that TGF- β 1 has a role in immunosuppression by modulating the activation, proliferation, and differentiation of T-cells (Letterio & Roberts, 1998). Further research has shown that TGF- β 1 is needed for the function of Foxp3⁺ T-regs (Marie, Letterio, Gavin, & Rudensky, 2005). However, more recent research has shown that TGF- β 1 is needed for the development of Th17 cells from naive T-precursors (Mangan et al., 2006, Qin et al., 2009). Here, our results corroborate the pro-Th17 activity of TGF- β 1 findings, observing increased blood CD4⁺CD39⁺ T-cells and pro-inflammatory cytokines in the vehicle group in conjunction with higher TGF- β 1 levels in the colon compared to the hemin treated group. In the mesenteric lymph nodes, IL-12 and IL-23 levels were significantly increased in the vehicle group again indicating an increase in Th17 load (Figure 36). Recent research that has found that IL-12 and IL-23 are primary cytokines in the development of Th17 cells (Teng et al., 2015). IL-13, also significantly increased in the vehicle group, is known to suppress inflammatory cytokine production, however, other research has shown that IL-13's suppressive capabilities are lessened in diseased patients and in addition, IL-13 is involved in the induction of TGF- β 1 secretion which we saw in this research (Fichtner-Feigl, Strober, Kawakami, Puri, & Kitani, 2005, KUCHARZIK et al., 1996, Wynn, 2003). Finally, the spleen saw significant cytokine changes in 10 of 12 cytokines evaluated (all except IL-17A and IL-4), with higher concentrations in the vehicle group which highlights the overall immunosuppressive

capabilities of hemin as a treatment leading to the effector subset (Th17) changes seen here (Figures 37 and 38). While IL-10 levels were significantly increased in the spleen, the fact that a suppressive cytokine is upregulated could simply indicate enhanced inflammation and disease which strengthens the case for hemin as an immunosuppressive treatment. Indeed, in the intestinal epithelium of CD patients IL-10 is significantly increased during inflammation in associated with IL-10 producing monocyte infiltrate (Marlow, 2013).

The HO-1 activity assay did not detect any changes in activity between groups, the primary value needed to identify an increase in HO-1 as a product of bilirubin production. This could be due to lack of sensitivity of the assay, or the limited quantity of tissue available for use. Additionally, in the transgenic strain RAG2^{-/-} mice there is likely to be different levels of expression of multiple factors in response to stress as a results of the absence of B or T-cells, other than the naive T-cells used to induce disease.

Study Limitations

While it is possible that treatment did not have a large effect on disease, the trends seen in the weight changes, AUC, endoscopy scores, and cytokine panels suggest an increase in group size would yield statistically significant differences. In addition to larger group sizes, additional groups could have been added, such as a naïve (no cell transfer) control and a Sn-protoporphyrin IX (SnPP) treatment group. SnPP, is a competitive inhibitor which also induces HO-1 but blocks enzyme activity (Zhang et al., 2014). Another limitation could be the hemin dose and/or frequency and duration of

dose. No toxic effects were observed, indicating that a larger or more frequent dose would be a possibility. Histopathology scoring, which requires a board certified pathologist and was outside of the budget could have been a more sensitive detection of disease than video endoscopy due to the low severity in the disease model compared to chemical models.

The HO-1 activity assay did not result in any usable data, this could be due to the sensitivity of the assay. Researchers who have used this in the past did not indicate mass of tissue required for the assay; a relatively small 1 cm piece was used here to allow for the remainder to be used for histopathology sectioning. Th17 cells were a main focus of these experiments, and IL-17A was used to infer an active Th17 response. However, the IL-17A ELISA yielded results below the limit of detection for many of the samples. It is possible that a more complete picture would be obtained by assessing the concentration of other IL-17 isoforms rather than solely focusing on IL-17A. Additionally, the lower T-cell load and complete absence of B-cells in the adoptive transfer model compared to a naïve wild type mouse could be a major factor influencing the concentrations of cytokines; C57Bl/6 mice have on average 60% lymphocytes in their blood compared to just over 10% seen here (JAX Physiological Data Summary - C57BL/6J, 2016). Finally, there are commercially available assays used to quantify Th17, HO-1, and T-Reg ELISAs, however limited resources precluded their use in this case. and other methods of detection were chosen as substitutes (Th17/TGF- β 1 multiplex, HO-1 activity assay, and FACS analysis).

Future Research Directions

Research building on these results should include additional T-cell markers for FACS analysis, as the field has become increasingly aware that subsets of effector T-cells can be differentiated from one another using multiple distinct cell markers. For example, CD161 combined with CD39 indicates a pathogenic Th17 cell, while Foxp3 with CD39 indicates a T-reg (Bai et al., 2014, Dwyer et al., 2010). Moreover, analyzing transcription factors Foxp3 and ROR- γ by FACS would allow further characterization of T-cells. Using an ELISA to detect HO-1 would be a more accurate and reliable method for detection and would likely require less sample, allowing for more effective analysis using that tissue. In the adoptive transfer model of colitis, disease typically presents less severely than in chemical models as shown by the peak score of 2 via endoscopy (Figure 21), as compared with an average peak score >2.5 in the DSS model ("Colitis/Inflammatory Bowel Disease (IBD) | Biomodels", 2016). Due to this, histopathological scoring by a trained pathologist may be essential to identify subtle inflammatory damage by morphological changes

An avenue to explore and expand upon these results would be to look at the role of integrins in Th17 development in this model using hemin as treatment. Research has shown that integrin $\alpha\text{v}\beta 8$ activation of TGF- $\beta 1$ is needed to induce Th17 differentiation (Pociask & Kolls, 2010). Here, we showed a significant increase in TGF- $\beta 1$ in colon tissue; it would be important to determine if integrins are being affected as part of treatment and/or as part of the overall immunosuppressive capabilities of hemin. Finally, the data presented suggest the adoptive transfer model is a prime candidate for future

research focusing on TGF- β 1 and T-cell development, which has reported conflicting results in the past. (Letterio & Roberts, 1998, Mangan et al., 2006, Marie, Letterio, Gavin, & Rudensky, 2005, Qin et al., 2009).

Conclusion

I hypothesized that hemin would upregulate HO-1 which then would have a powerful effect in diminishing disease determined by weight loss and video endoscopy outcomes and this effect would be associated with decreased pro-inflammatory cytokines and Th17 cell differentiation. Data from the adoptive transfer induced colitis model is presented showing significant decreases in CD4⁺ and CD4⁺CD39⁺ T-cells counts and pro-inflammatory cytokines IFN- γ and TNF- α together with positive trends in weight change, video endoscopy score, and other cytokines in the hemin treatment group. HO-1 was unable to be detected and therefore cannot be positively identified as the treatment target. Additionally, since no T-reg population was identified it does not appear that hemin induces the proliferation of T-regs from naïve T-cells. Together, these data strongly suggest that hemin acts as an anti-inflammatory agent with T-cell modulatory effects.

Appendix

Group Number	Number of Animals	Transfer (Day 0)	RO Bleed for FACS	Dose	Weight Blood Diarrhea Checks	Endoscopy	Sacrifice
Donor	10 male C57Bl/6	Harvest Spleens	--	--	--	--	--
1	8 males (Rag2 ^{-/-})	0.5x10 ⁶ CD62L+CD44- Cells	Day 14, 28, 35, and 56	Vehicle	0-56	Day 14, 21, 28, 35, 42, 49 and 56	Day 28 n=3 Day 56 n=5
2	8 males (Rag2 ^{-/-})	0.5x10 ⁶ CD62L+CD44- Cells	Day 14, 28, 35, and 56	Hemin 50ug/kg	0-56	Day 14, 21, 28, 35, 42, 49 and 56	Day 28 n=3 Day 56 n=5

Table 1. In-Life Study Design: 10 donor mice provided naïve CD4⁺ T-cells to induce colitis in 16 RAG2^{-/-} mice. Collections, measurements, and dosing provided valuable data on disease progression. Sacrifice endpoints were used to detect changes in tissue samples colon, spleen, and mesenteric lymph nodes.

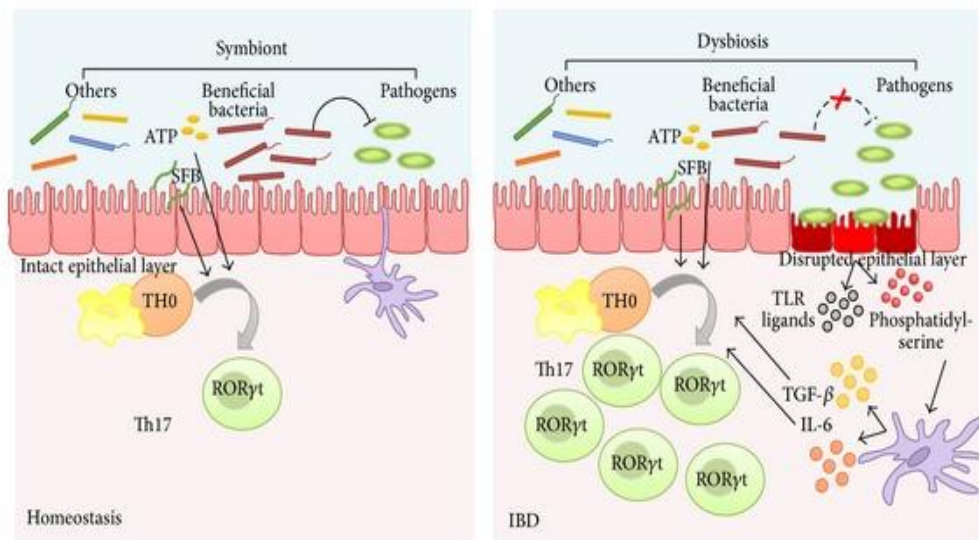


Figure 1. Insult to gut results in loss of bacterial balance and dysregulated immune response. Beneficial bacteria no longer provide protection against pathogens leading to an inflammatory cascade marked by Th17 proliferation (Gálvez, 2014).

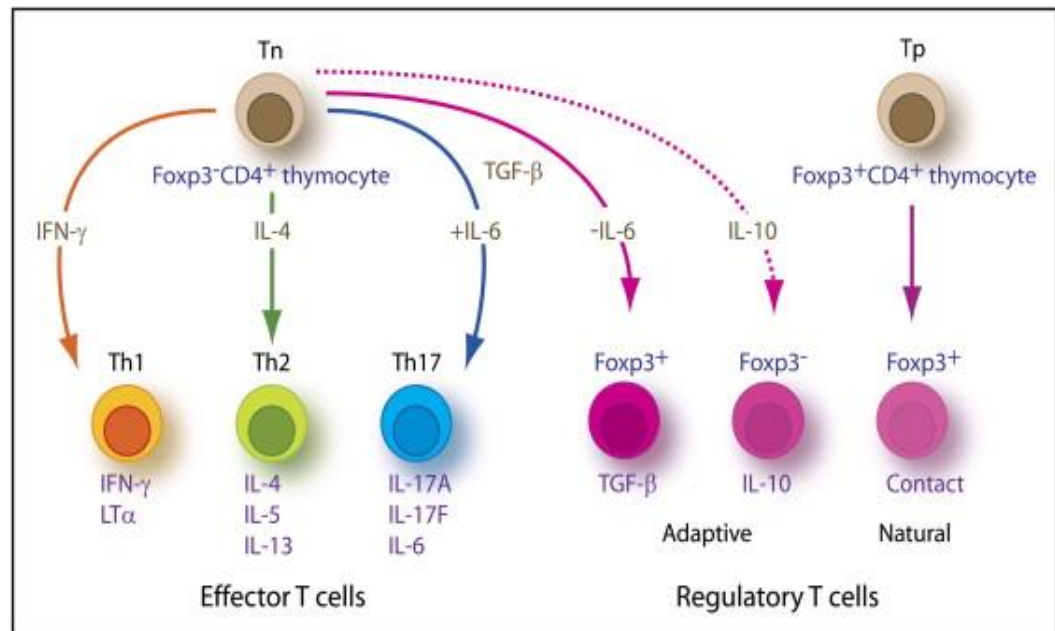


Figure 2. Naïve T-cells “Tn” have the potential to become many effector and T-regs. The cytokine milieu present drives production of specific subsets (Weaver, Harrington, Mangan, Gavrieli, & Murphy, 2006).

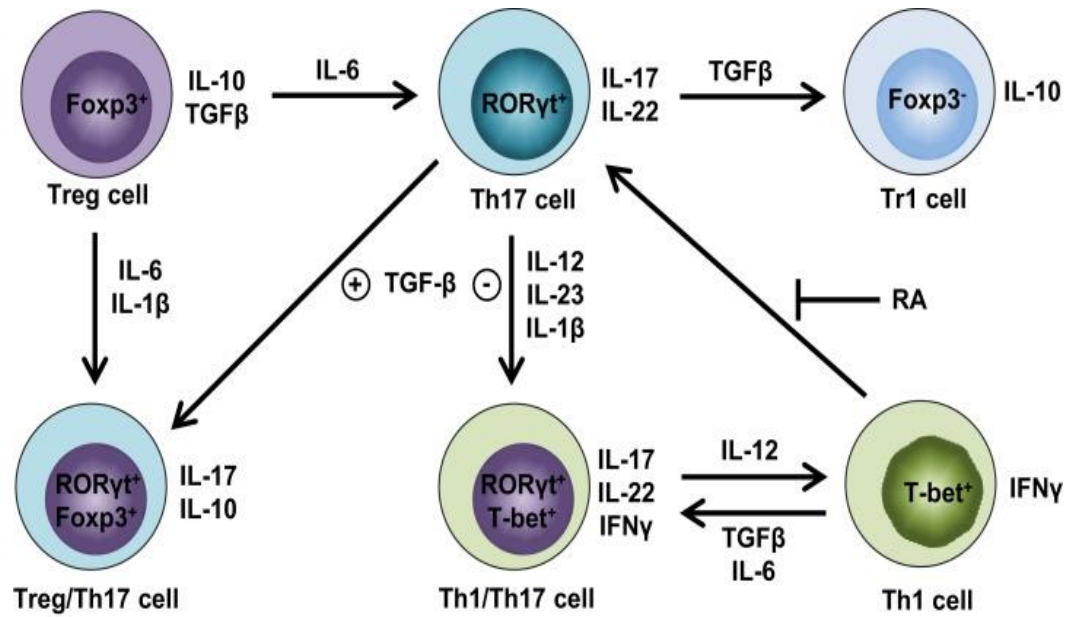


Figure 3. The complex relationship between T-reg and Th17 plays a significant role in the pathogenesis of IBD and highlights the importance in understanding the characteristics of each subset (Omenetti & Pizarro, 2015).

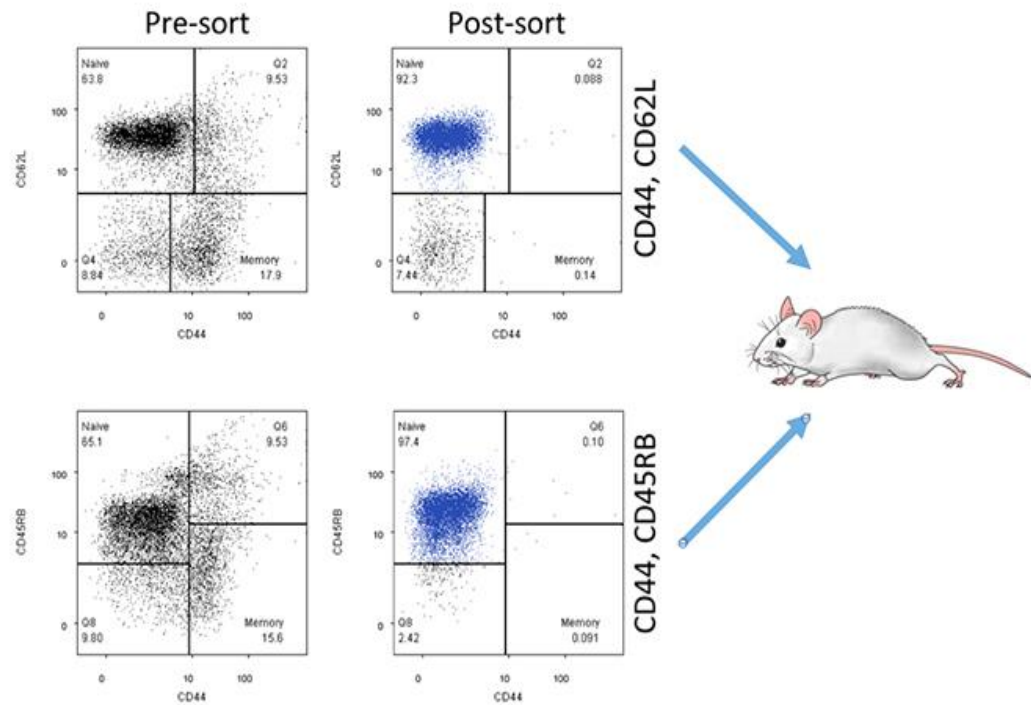


Figure 4. The magnetic cell sort for CD62L+CD44- and the previously described CD45+CD25- T-cell sort express naïve T-cell marker CD45RB+ as shown by FACS analysis. These cells are then resuspended and injected in mice to induce colitis ("Colitis/Inflammatory Bowel Disease (IBD) | Biomodels", 2016).

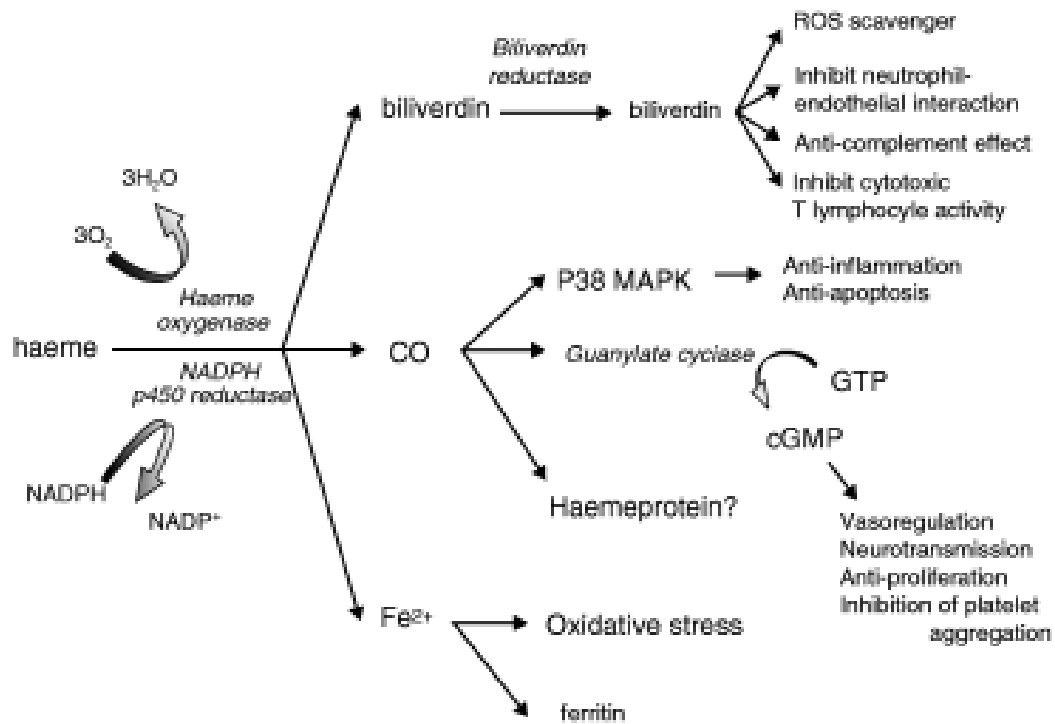


Figure 5. Degradation of heme by HO-1 produces anti-inflammatory byproducts biliverdin, carbon monoxide, and Fe²⁺. These products lead to several anti-inflammatory effects such as ROS scavenging, neutrophil infiltrate inhibition, and anti-apoptosis (Naito, Takagi, & Yoshikawa, 2004).

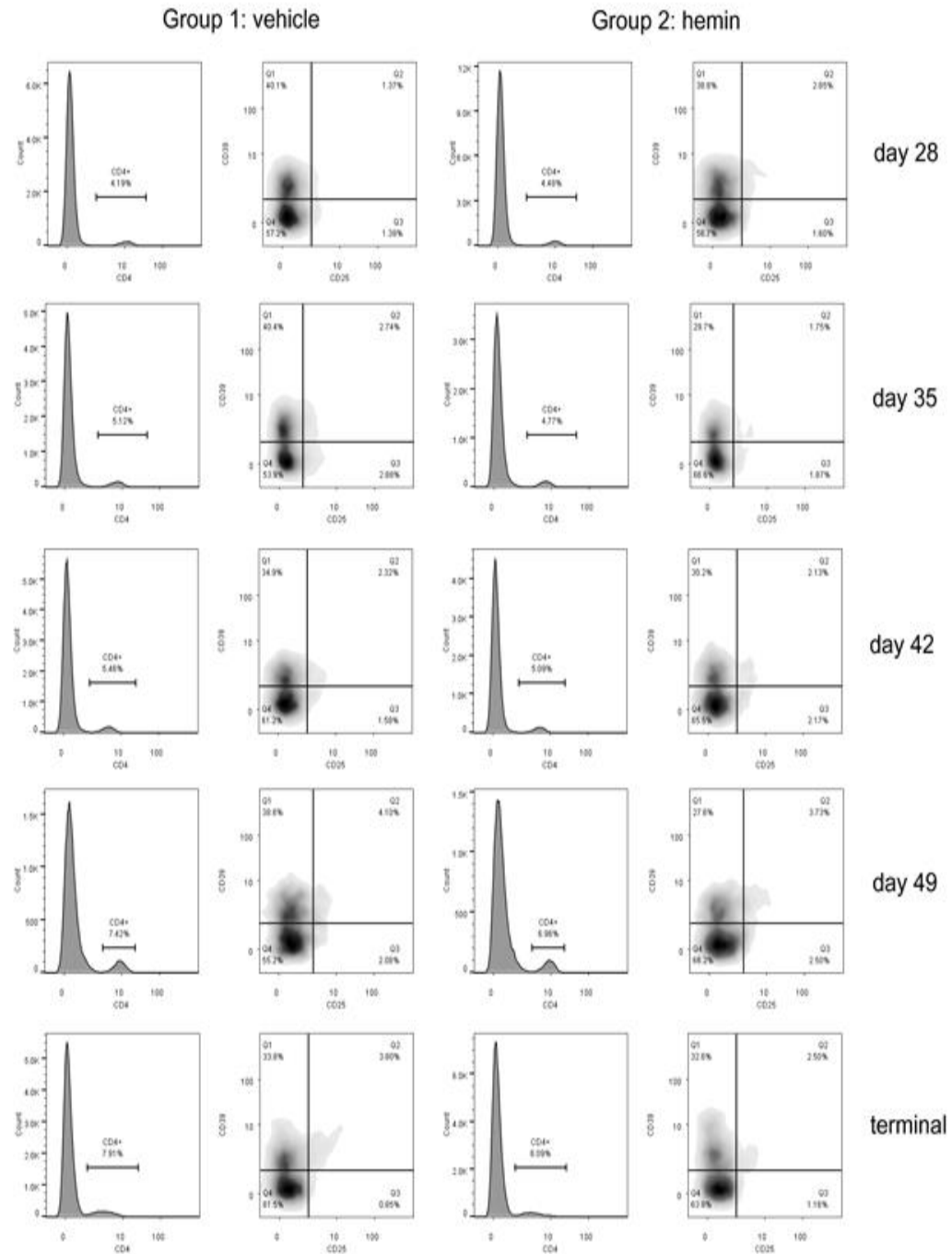


Figure 6. Representative Blood FACS analysis measuring CD4+ T-cell counts and Plots which gated for CD39+ (Th17) on the Y-axis, and CD25+ (T-reg) on the X-axis. Blood was collected at timepoints detailed in table 1 and processed for FACS analysis. No CD25+ populations were found, but CD39+ populations are present but significantly smaller in the hemin treatment group at each timepoint.

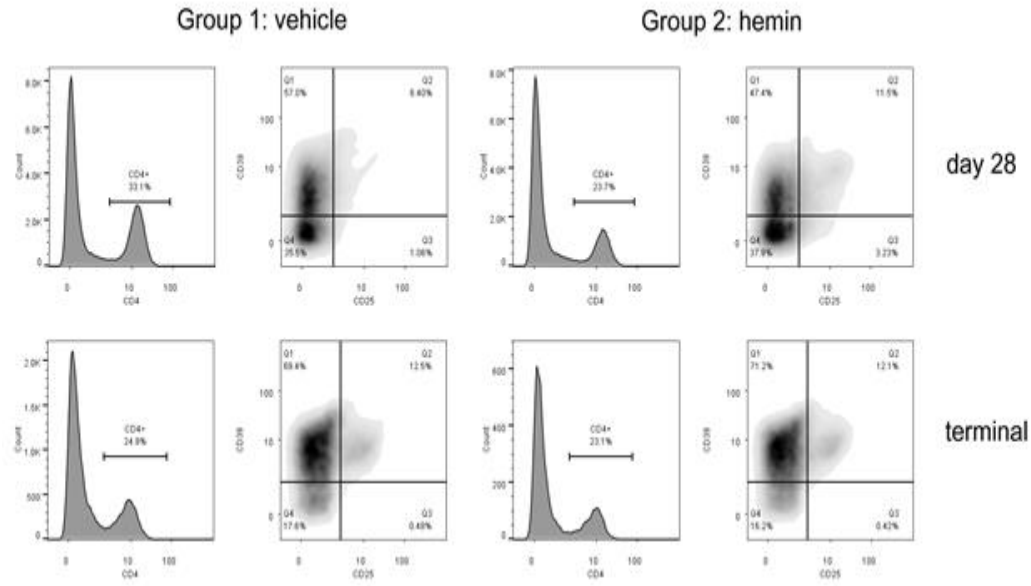


Figure 7. Representative Mesenteric Lymph Node FACS analysis measuring CD4+ T-cell counts and Plots which gated for CD39+ on the Y-axis, and CD25+ on the X-axis. Tissue was collected at timepoints detailed in table 1 and processed for FACS analysis. No CD25+ populations were seen but CD39+ populations are higher compared to blood, but no significant differences were found.

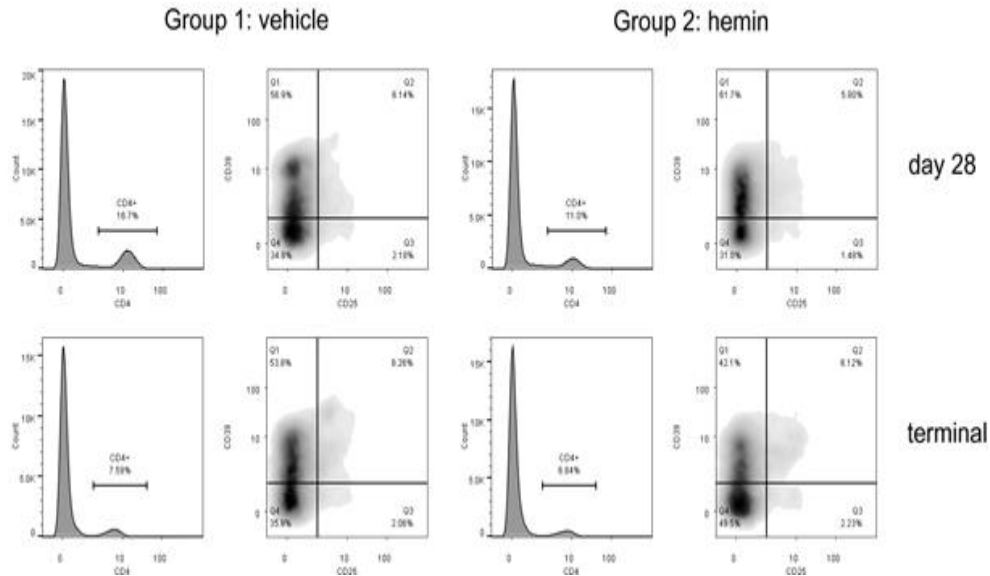


Figure 8. Representative Spleen FACS analysis measuring CD4+ T-cell counts and Plots which gated for CD39+ on the Y-axis, and CD25+ on the X-axis. Tissue was collected at timepoints detailed in table 1 and processed for FACS analysis. Similarly to the mesenteric lymph nodes, no CD25+ populations were seen but CD39+ populations are higher compared to blood, but no significant differences were found.

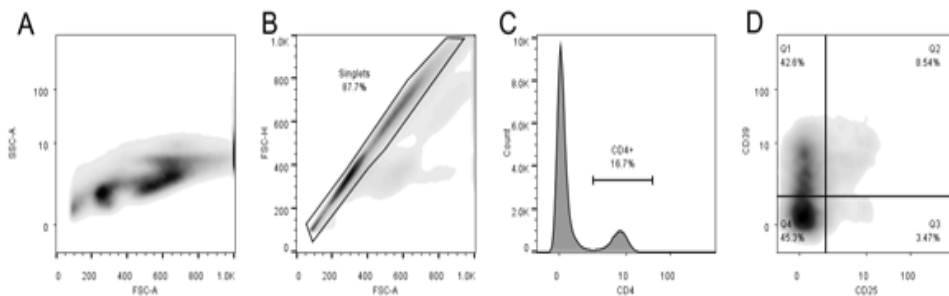


Figure 9. FACS Gating strategy. Panel A is a side and forward scatter plot displaying overall cell size. Panel B is a side and forward scatter plot to isolate single cells and remove debris and any even ts that are not a single cell. Panel C is counting populations of CD4+ T-cells. Panel D is gated for CD39+ on the Y-axis and CD25+ on the X-axis.

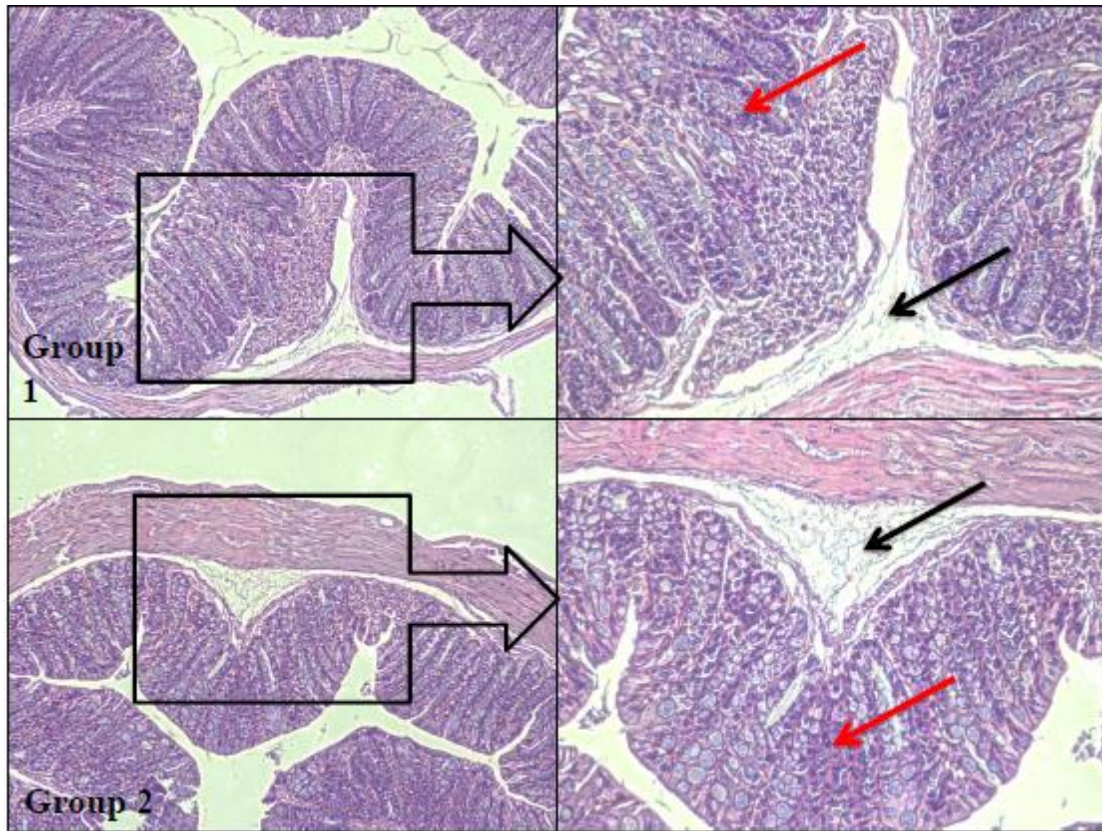


Figure 10: Example Day 28 Histopathology analysis on colon sample shows sufficient disease induction. Panel A/C are a 100x magnification and Panel B/D are 200x. Black arrows indicate areas of increased edema and red arrows indicate areas of monocyte infiltrate indicating mild to moderate disease severity.

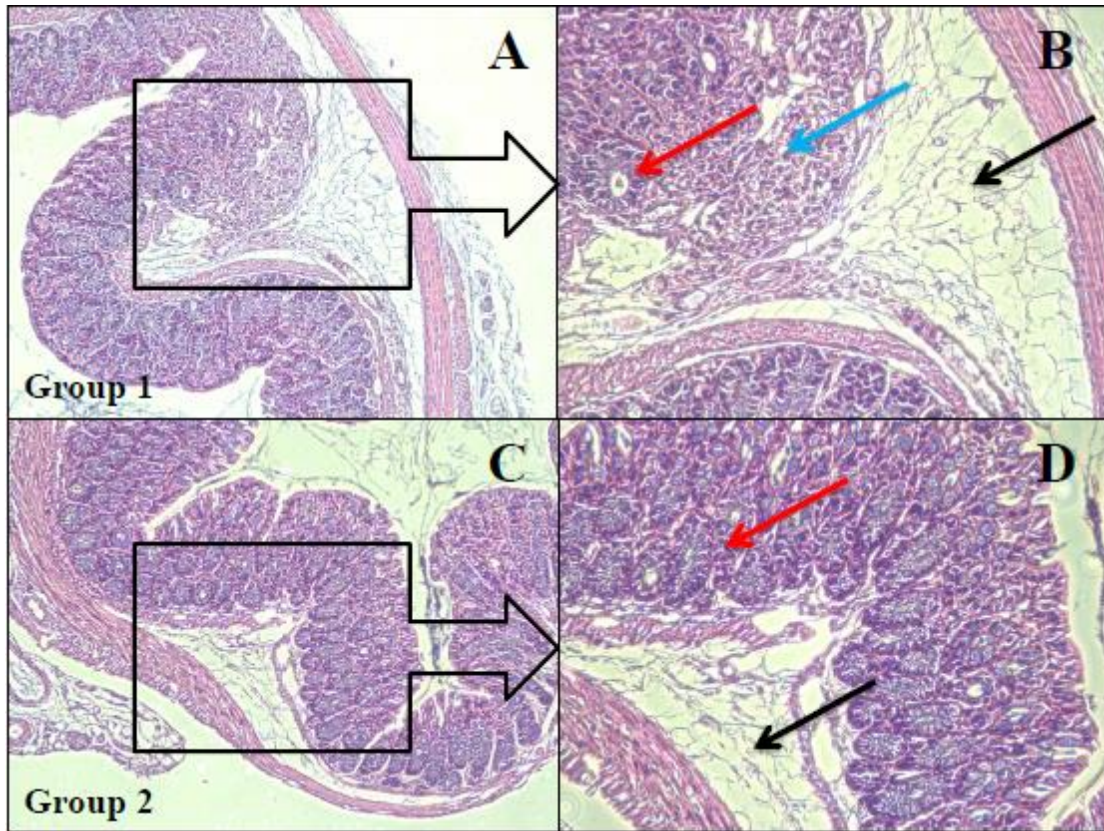


Figure 11: Example Day 56 Histopathology analysis on colon sample shows disease progress and severity. Panel A/C are a 100x magnification and Panel B/D are 200x. Black arrows indicate areas of increased edema, red arrows indicate areas of monocyte infiltrate, and blue arrows indicate areas where crypt morphology is perturbed indicating moderate to high disease severity.

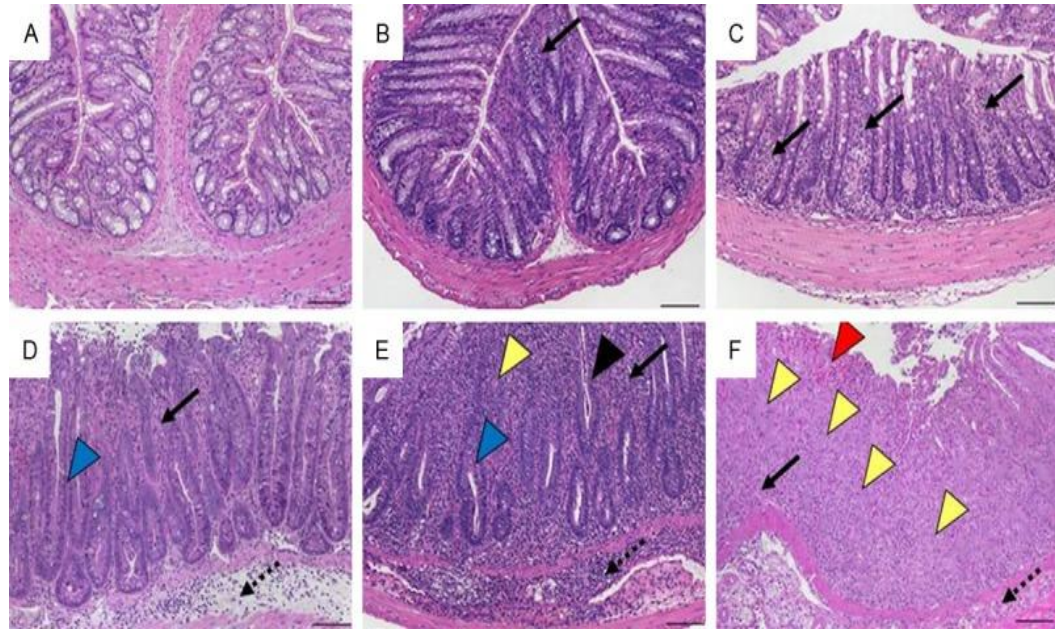


Figure 12: “Histomorphology of colon tissue after transfer of CD4⁺CD45RB^{hi} T cells into Rag1^{-/-} mice representing scores according to scheme 3 (adoptive transfer colitis scale). A. Score 0: normal colon mucosa with intact epithelium; B. Score 1: scattered inflammatory cell infiltrates in the mucosa; C. Score 2: diffuse mucosal infiltrates without submucosal spreading and intact epithelial layer; D. Score 3: moderate infiltration of inflammatory cells into mucosa and submucosa with epithelial hyperplasia and goblet cell loss; E. Score 4: marked inflammatory cell infiltrates in mucosa and submucosa accompanied by crypt abscesses and loss of goblet cells and crypts; F. Score 5: marked inflammatory cell infiltrates within the mucosa spreading to the submucosa going along with crypt loss and hemorrhage. Original magnification $\times 100$; scale bars 100 μm ; arrows-inflammatory cell infiltrates within mucosa (solid) and submucosa (dotted); black arrowhead-crypt abscess, blue arrowhead-goblet cell loss; yellow arrowhead-crypt loss; red arrowhead-hemorrhage.” (Erben et al., 2014)

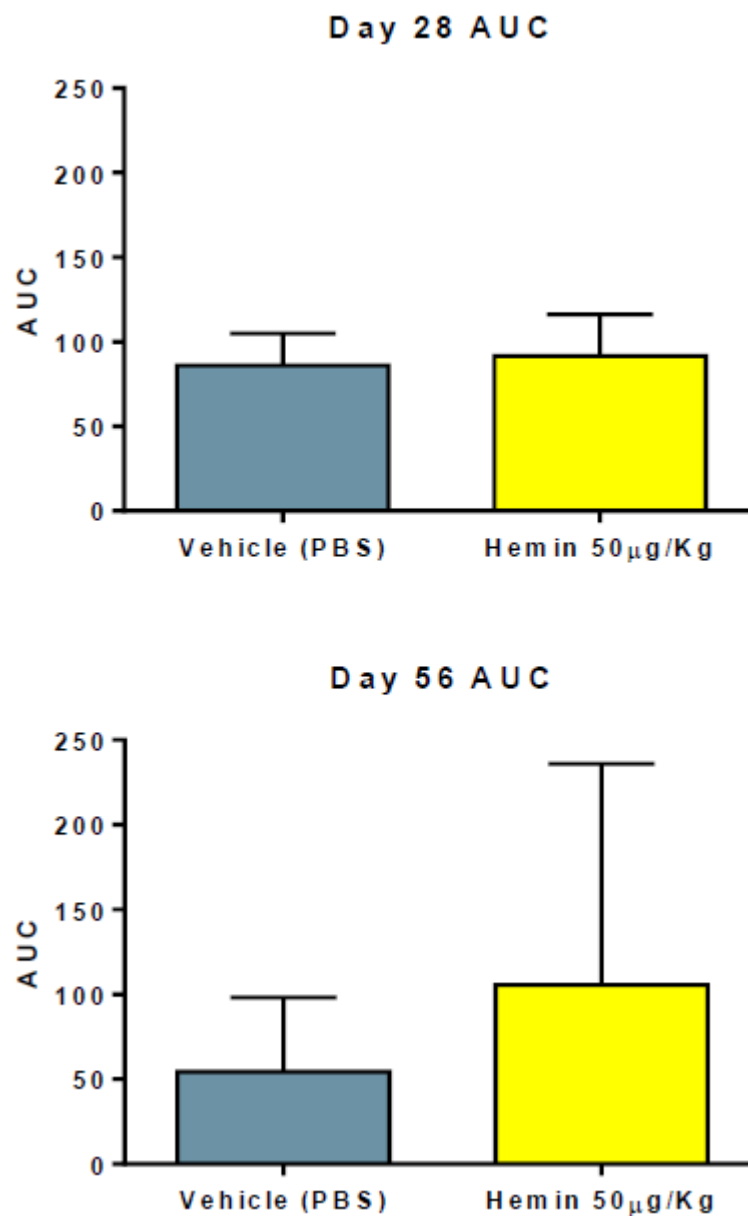


Figure 13. Weight changes represented by Area Under the Curve (AUC). Animal weights were measured daily throughout the study. Data is represented as group average \pm SEM. n=3 at day 28, n=5 at day 56 for group 1 and n=4 on day 56 for group 2.

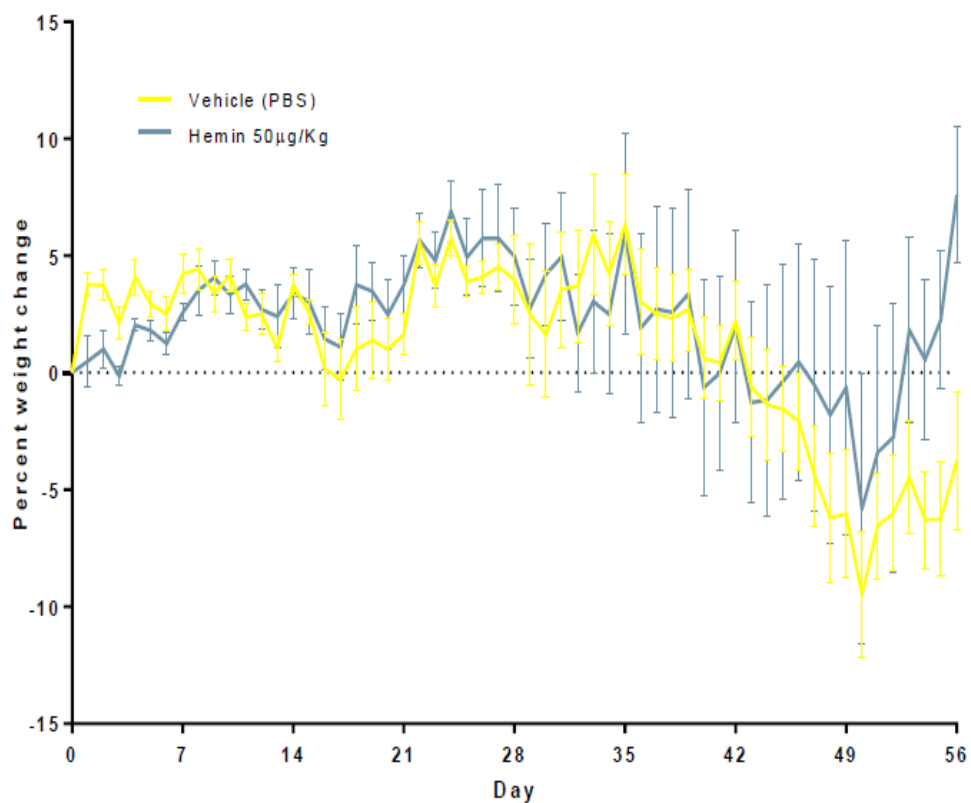


Figure 14. Mean Group Weight Change. Yellow = Vehicle, Blue= Hemin. Animal weights were measured daily throughout the study. Percent weight change was calculated relative to the starting weight on day 0. Data is represented as group average \pm SEM. n=8 per group.

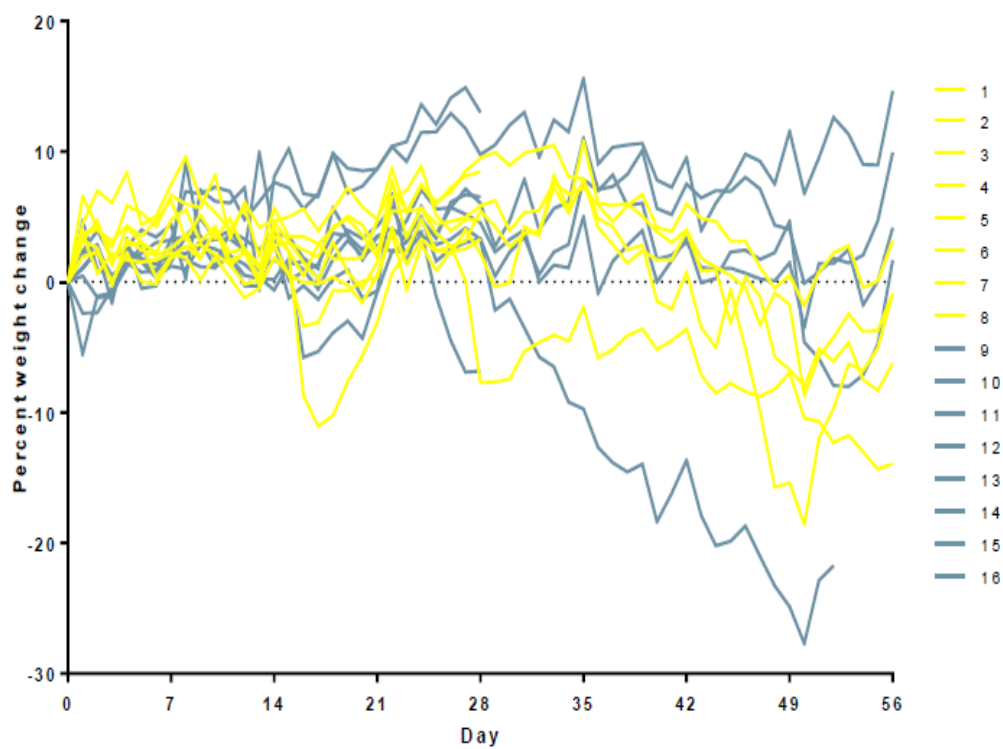


Figure 15. Individual Weight Change. Yellow = Vehicle, Blue= Hemin. Animal weights were measured daily throughout the study. Percent weight change was calculated relative to the starting weight on day 0.

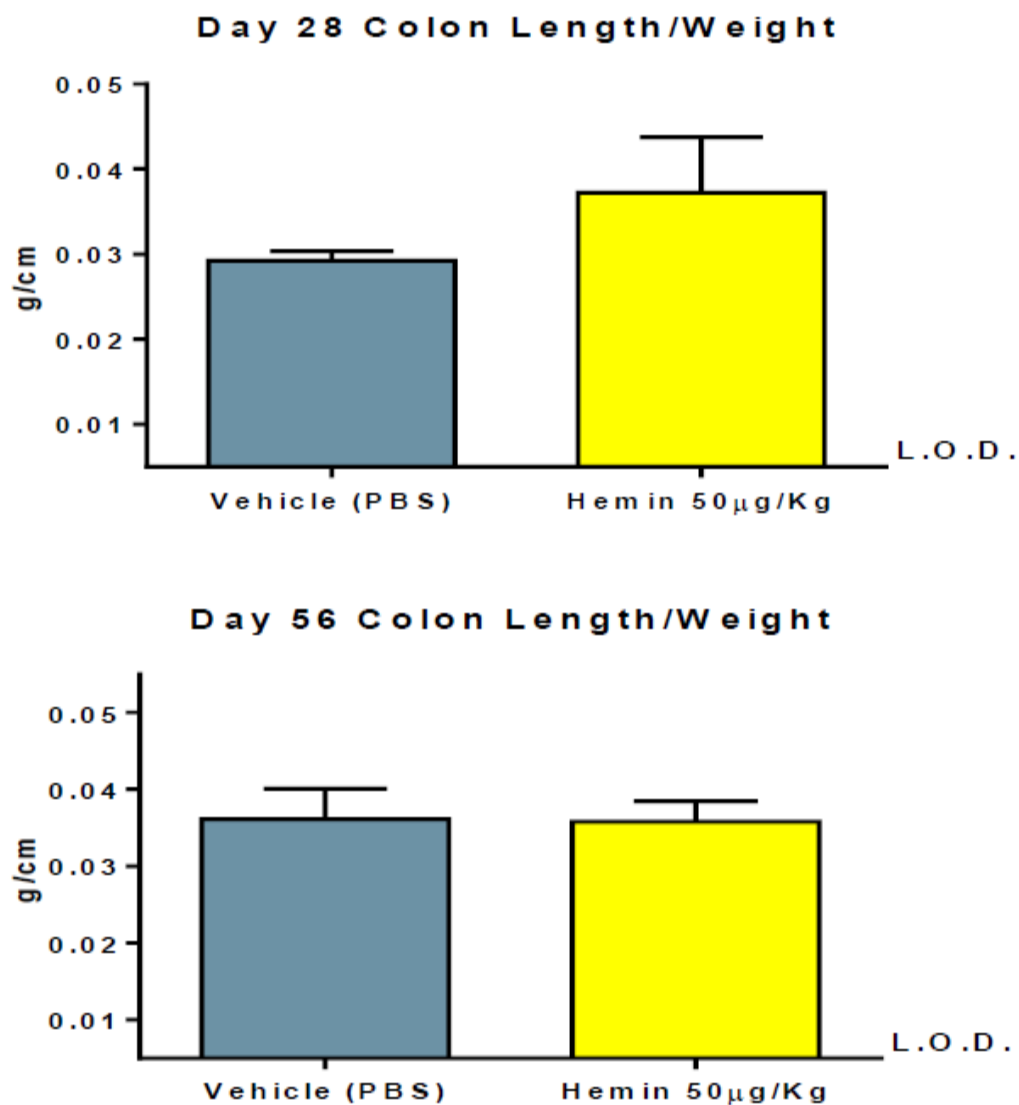


Figure 16. Mean Group Colon Length/Weight (g/cm). Colon was excised at sacrifice on day 28 and 56, flushed with saline and measured for weight and length. Data is represented as group average \pm SEM. n=3 at day 28, n=5 at day 56 for group 1 and n=4 on day 56 for group 2.

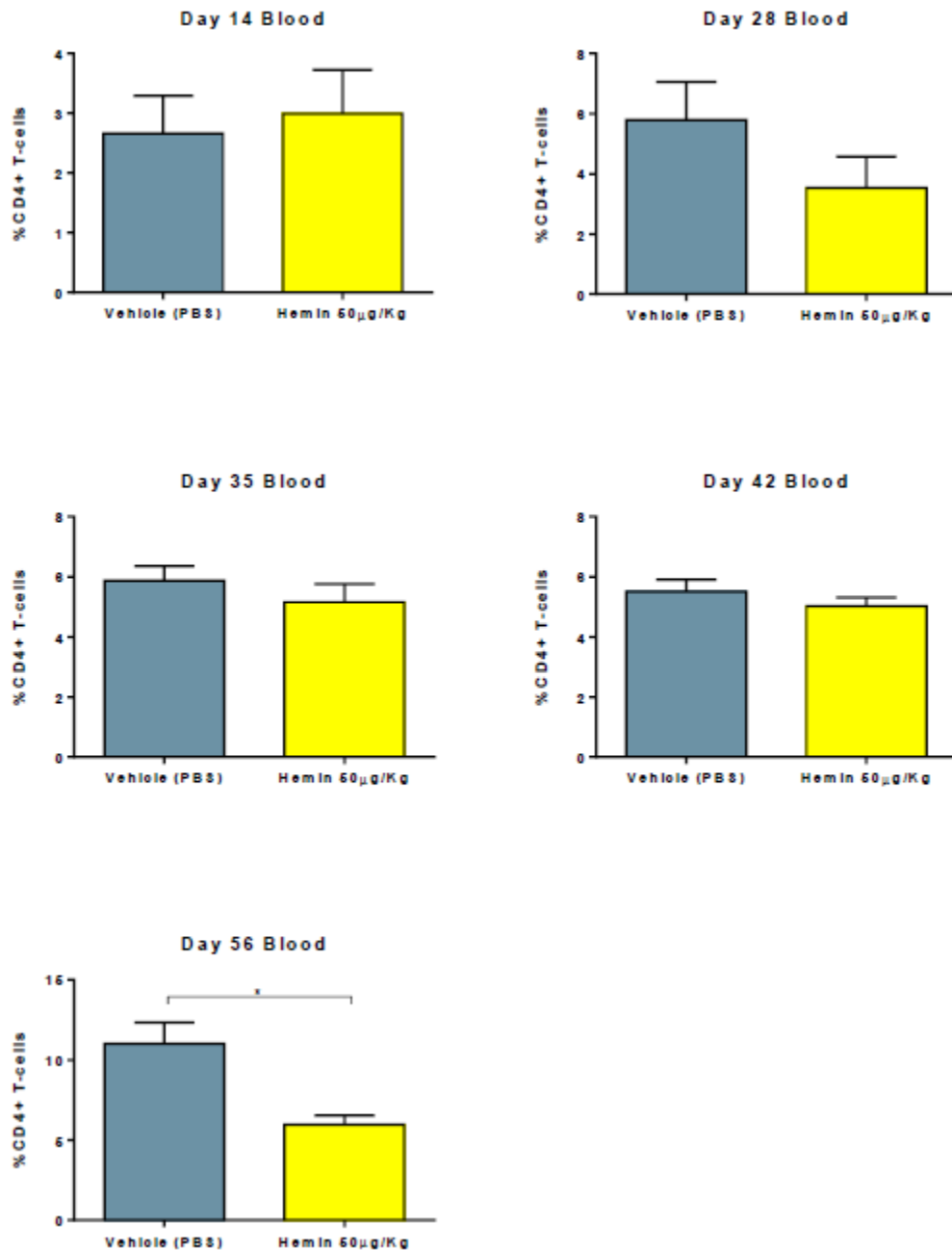


Figure 17. Mean blood CD4+ T-cell percentages. Blood was collected at timepoints in table 1 and processed for FACS analysis of CD4 expression. Data is represented as group average \pm SEM. n=8 per group on days 14 and 28, n=5 per group on days 35 and 42, and n=5 at day 56 for group 1 and n=4 on day 56 for group 2. *: p<0.05 as assessed by one tailed Student's T test.

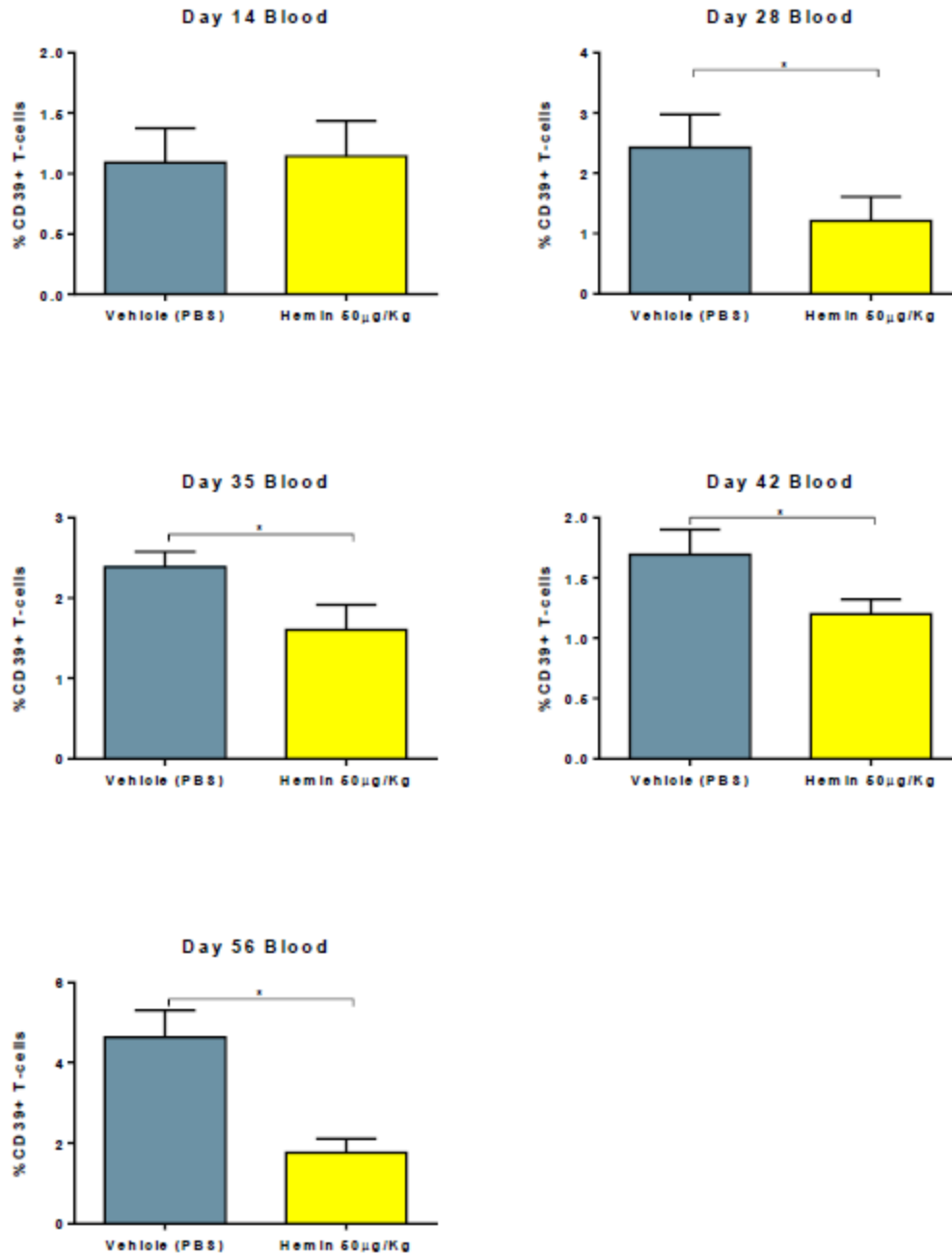


Figure 18. Mean blood CD4+CD39+ T-cell percentages. Blood was collected at timepoints in table 1 and processed for FACS analysis of CD4 and CD39 expression. Data is represented as group average \pm SEM. n=8 per group on days 14 and 28, n=5 per group on days 35 and 42, and n=5 at day 56 for group 1 and n=4 on day 56 for group 2. *: p<0.05 as assessed by one tailed Student's T test.

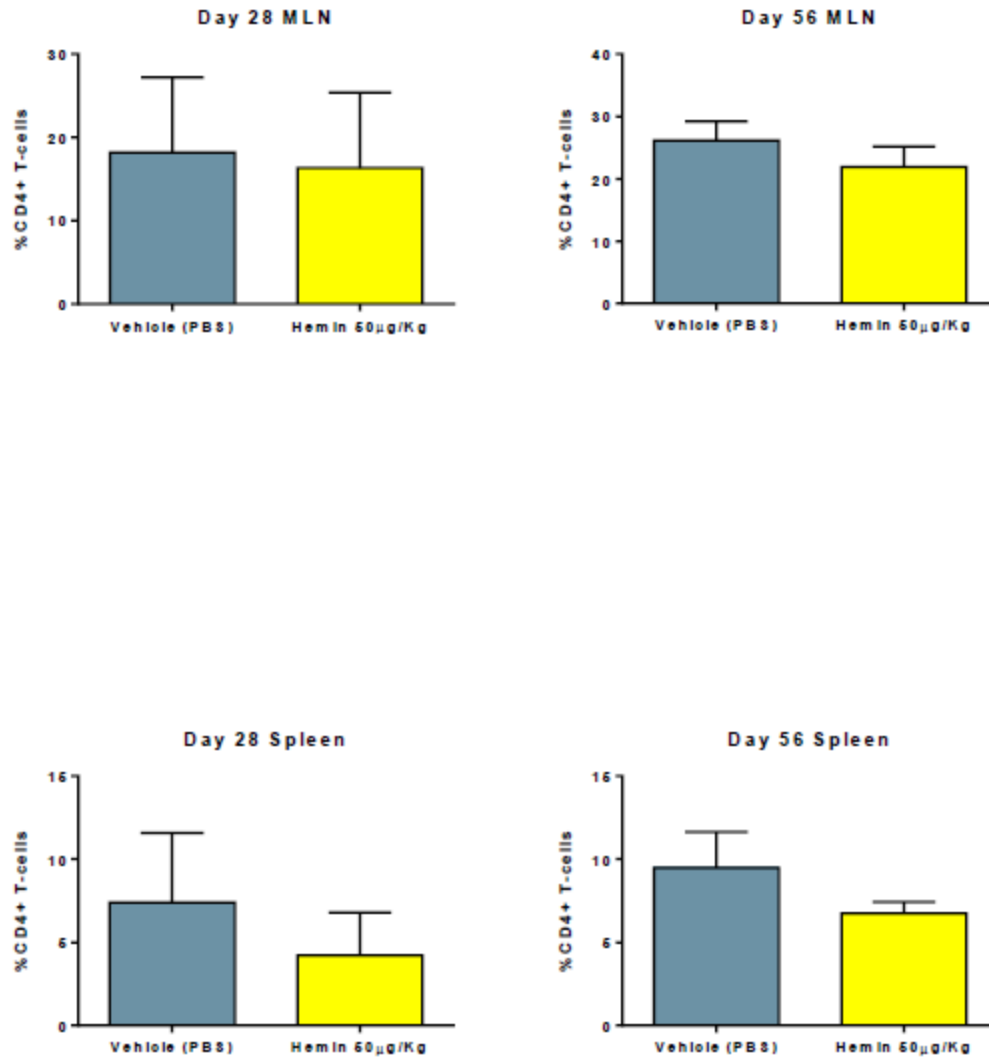


Figure 19. Mean tissue CD4+ T-cell. Tissue was collected at timepoints in table 1 and processed for FACS analysis of CD4 expression. Data is represented as group average \pm SEM. n=3 on day 28, and n=5 at day 56 for group 1 and n=4 on day 56 for group 2.

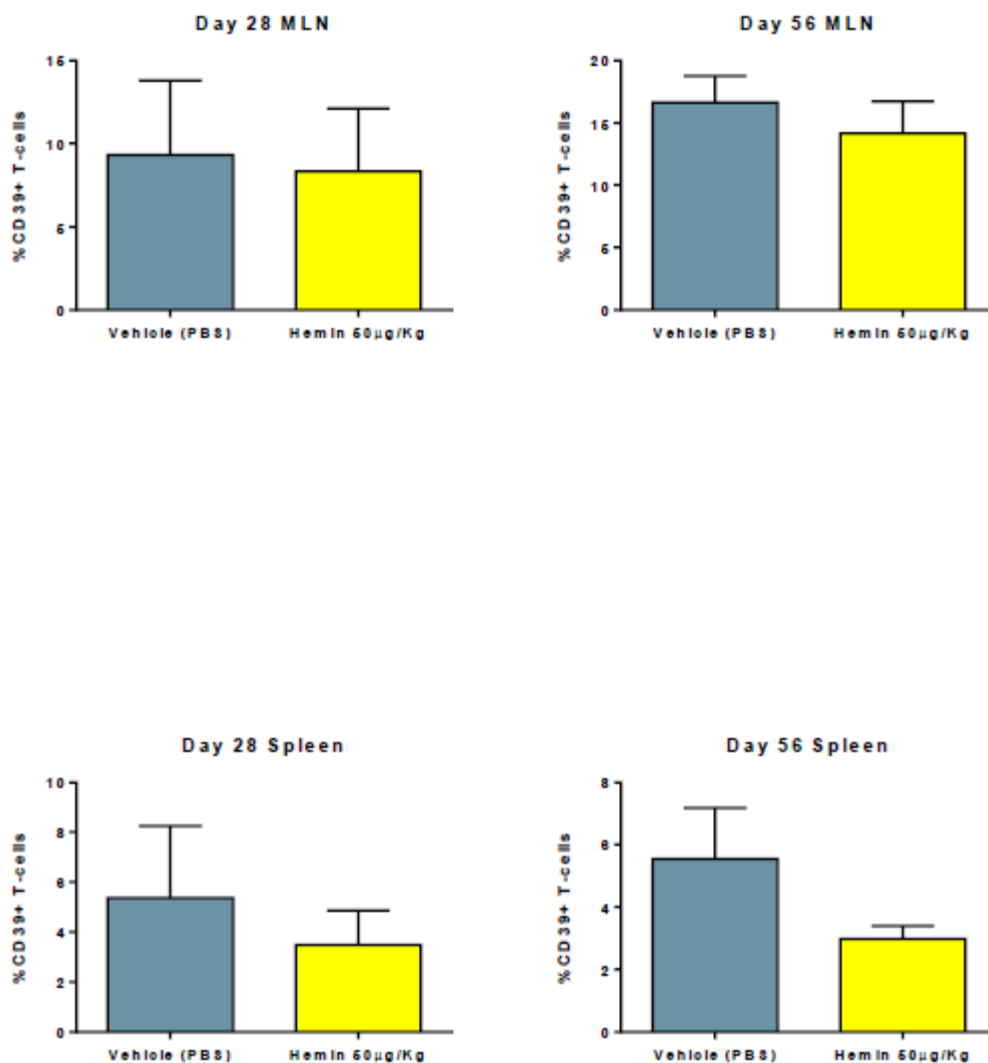


Figure 20. Mean tissue CD4+ CD39+ T-cell. Tissue was collected at timepoints in table 1 and processed for FACS analysis of CD4 and CD39 expression. Data is represented as group average \pm SEM. n=3 on day 28, and n=5 at day 56 for group 1 and n=4 on day 56 for group 2.

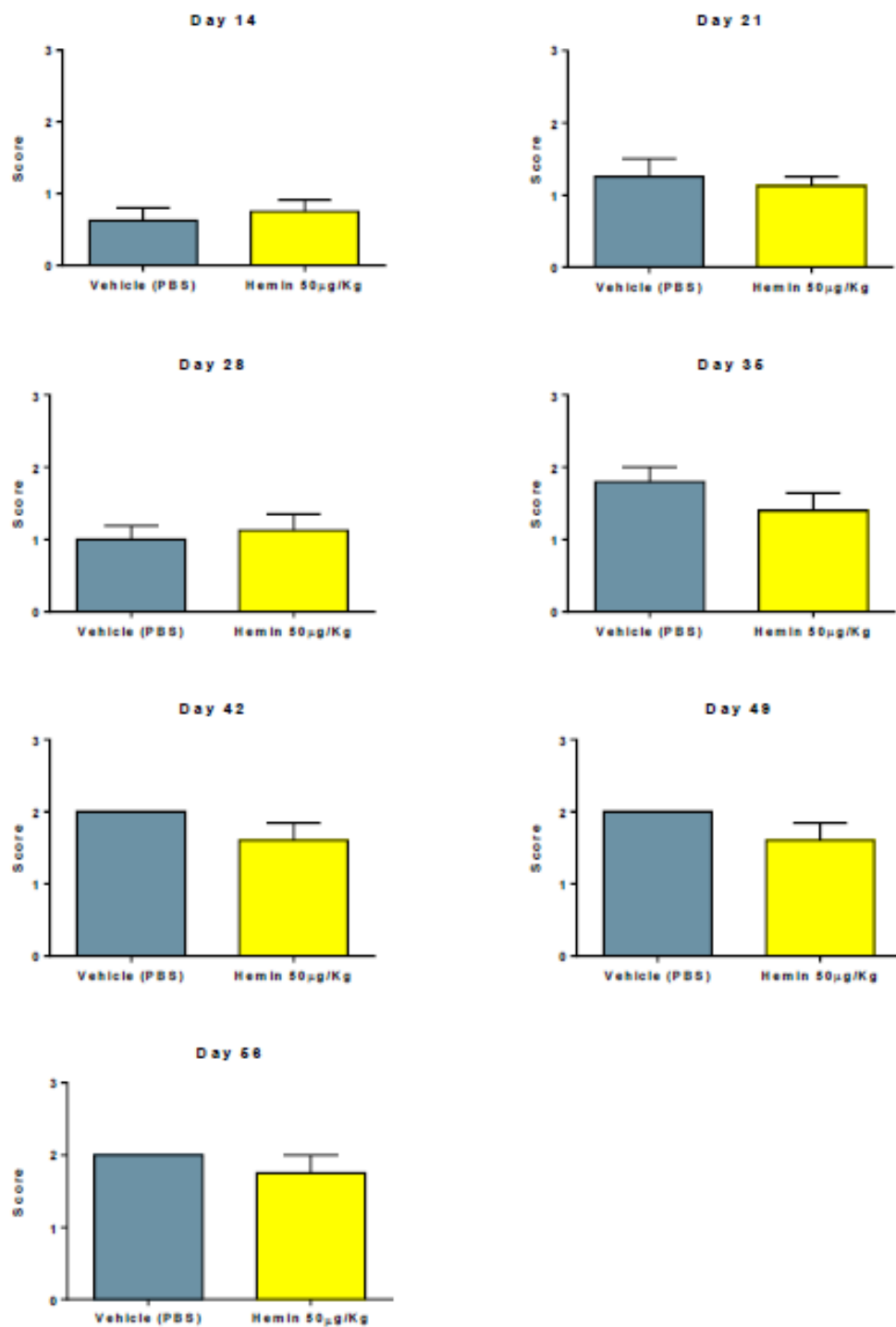
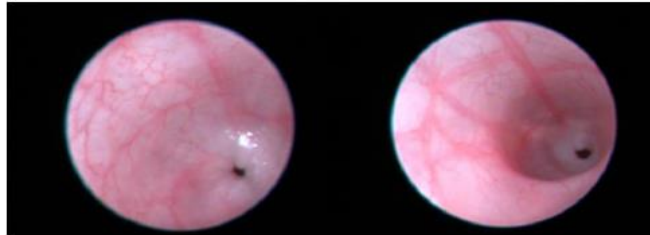


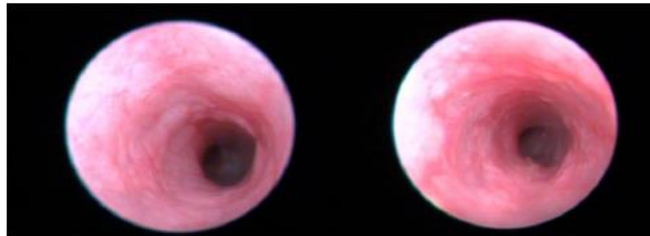
Figure 21. Mean video endoscopy scores. Colitis was assessed by video endoscopy at the timepoints indicated in table 1 and scored using the colitis scoring scale shown in Figure 10. Data is represented as group average \pm SEM. n=8 per group on days 14, 21, and 28, n=5 per group on days 35, 42, and 49 and n=5 at day 56 for group 1 and n=4 on day 56 for group 2.

Colitis Scoring Scale

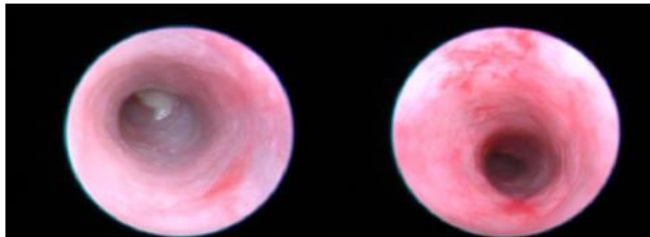
Score = 0; Naïve: no edema, mucosal sloughing, has clear vascularity.



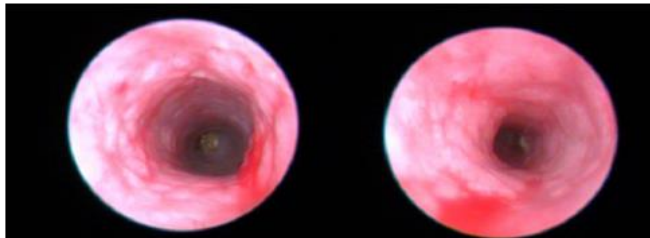
Score = 1; Edema, mucosal sloughing, decreased vascularity.



Score = 2; Edema, mucosal sloughing, decreased vascularity, friability.



Score = 3; Active bleeding, erosion.



Score = 4; Active ulceration, erosion.

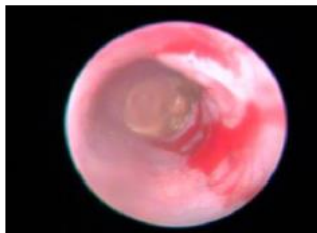


Figure 22. Video Endoscopy Scoring Scale developed by Biomodels, LLC with descriptions for each score. Escalating from a score of 0-4 disease progression is identified by changes in vascularity, edema, bleeding, erosion, and ulceration (Hamilton et al., 2011, Lyng, Stevens, Gordon, Watkins, & Sonis, 2008)

Day 14 Representative Endoscopy Images



Group 1 (Average Score =0.63)



Group 2 (Average Score =0.75)

Day 21 Representative Endoscopy Images



Group 1 (Average Score =1.25)



Group 2 (Average Score =1.13)

Figure 23. Day 14 and Day 21 representative endoscopy images. Video endoscopy performed at timepoints indicated in table 1 and pictures were taken of the most diseased area. Group averages are shown for comparison purposes. n=8 per group

Day 28 Representative Endoscopy Images



Group 1 (Average Score =1.00)



Group 2 (Average Score =1.13)

Day 35 Representative Endoscopy Images



Group 1 (Average Score =1.80)



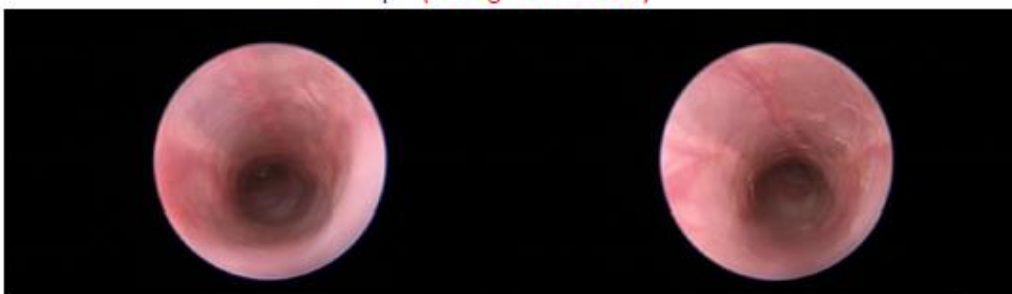
Group 2 (Average Score =1.40)

Figure 24. Day 28 and Day 35 representative endoscopy images. Video endoscopy performed at timepoints indicated in table 1 and pictures were taken of the most diseased area. Group averages are shown for comparison purposes. n=8 per group on day 28 and n=5 on day 35.

Day 42 Representative Endoscopy Images

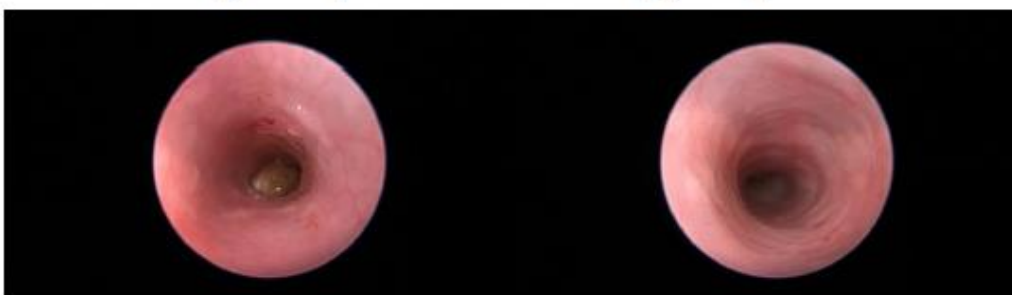


Group 1 (Average Score =2.00)

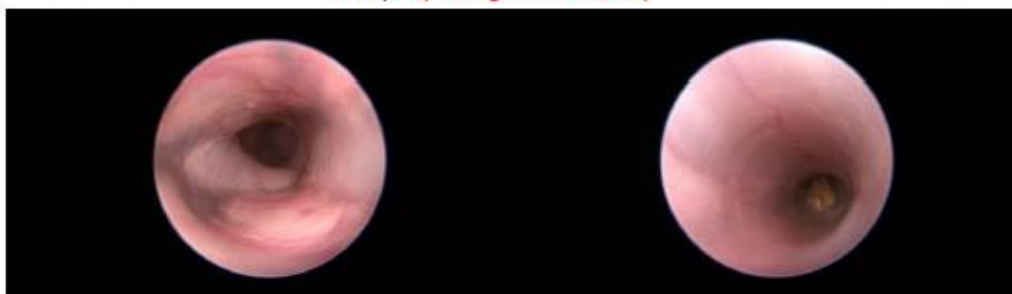


Group 2 (Average Score =1.60)

Day 49 Representative Endoscopy Images



Group 1 (Average Score =2.00)



Group 2 (Average Score =1.60)

Figure 25. Day 42 and Day 49 representative endoscopy. Video endoscopy performed at timepoints indicated in table 1 and pictures were taken of the most diseased area. Group averages are shown for comparison purposes. n=5 per group

Day 56 Representative Endoscopy Images



Group 1 (Average Score =2.00)



Group 2 (Average Score =1.75)

Figure 26. Day 56 representative endoscopy images. Video endoscopy performed at timepoints indicated in table 1 and pictures were taken of the most diseased area. Group averages are shown for comparison purposes. n=5 for group 1 and n=4 for group 2.

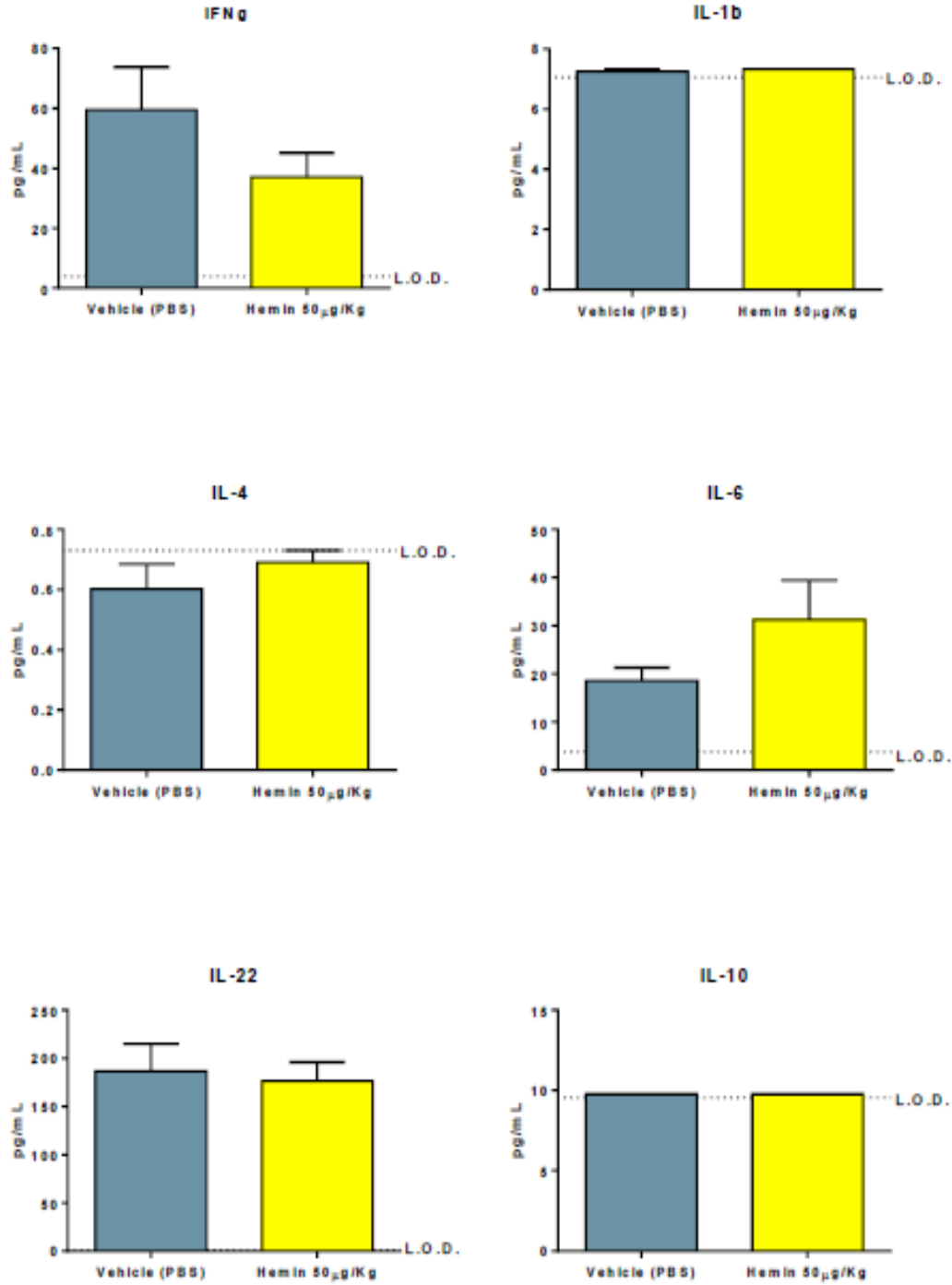


Figure 27. Day 28 Plasma Multiplex Graph Part 1. Plasma was collected at the timepoints indicated in Table 1. Samples were analyzed using multiplex ELISA. Data is represented as group average \pm SEM. n=8 per group. LOD: Limit of detection.

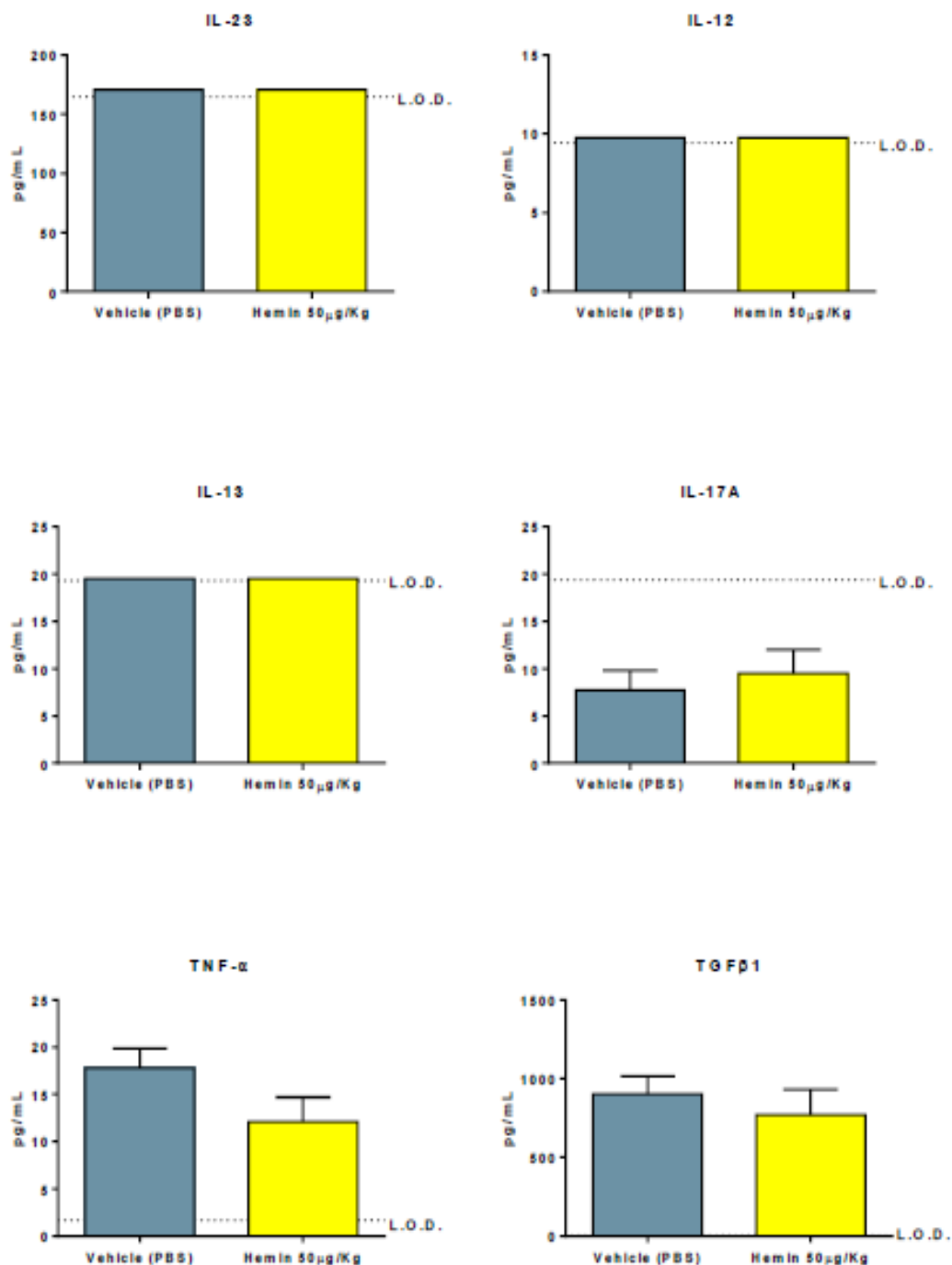


Figure 28. Day 28 Plasma Multiplex Graph Part 2. Plasma was collected at the timepoints indicated in Table 1. Samples were analyzed using multiplex ELISA. Data is represented as group average \pm SEM. n=8 per group. LOD: Limit of detection.

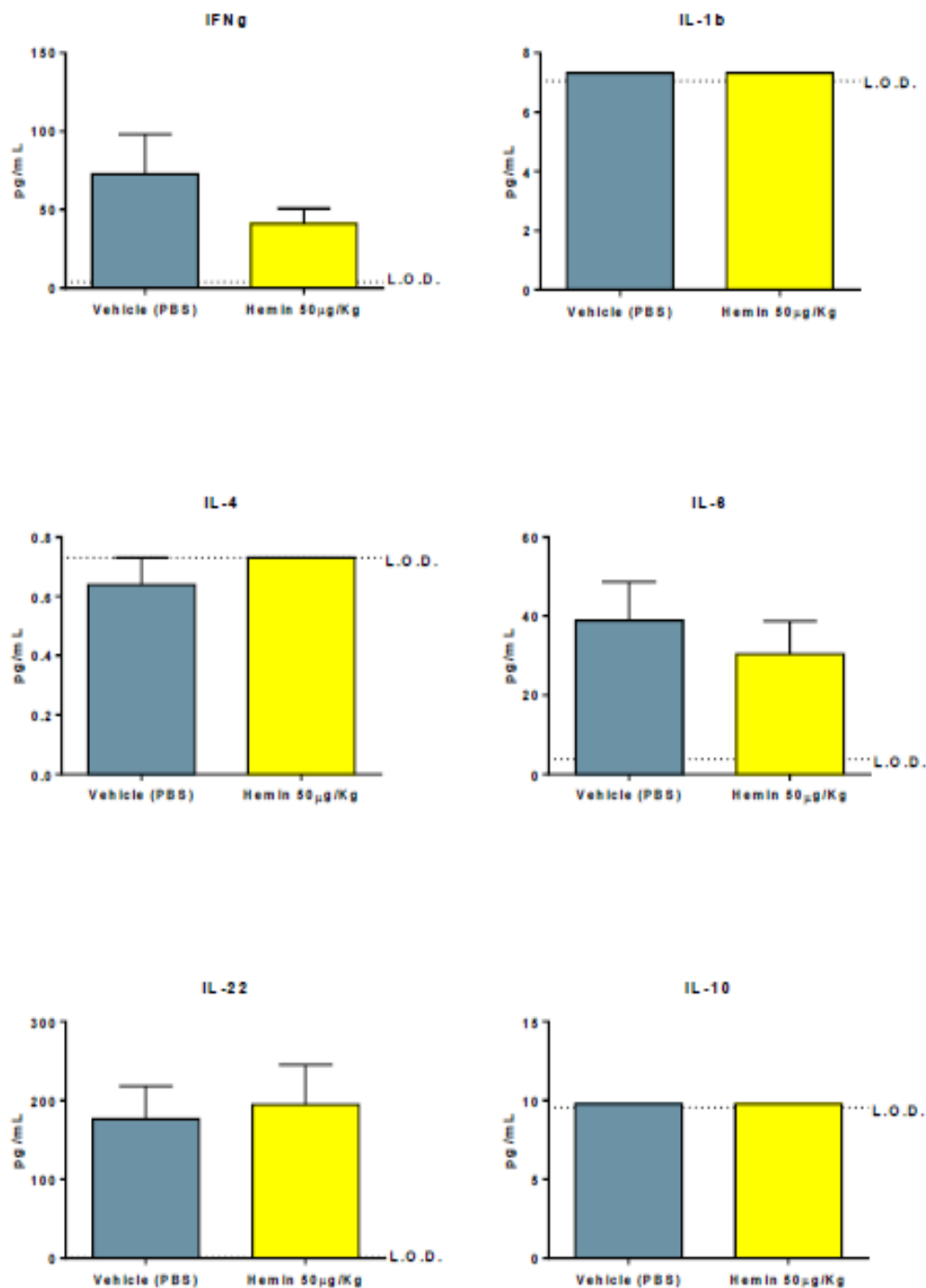


Figure 29. Day 35 Plasma Multiplex Graph Part 1. Plasma was collected at the timepoints indicated in Table 1. Samples were analyzed using multiplex ELISA. Data is represented as group average \pm SEM. n=8 per group. LOD: Limit of detection.

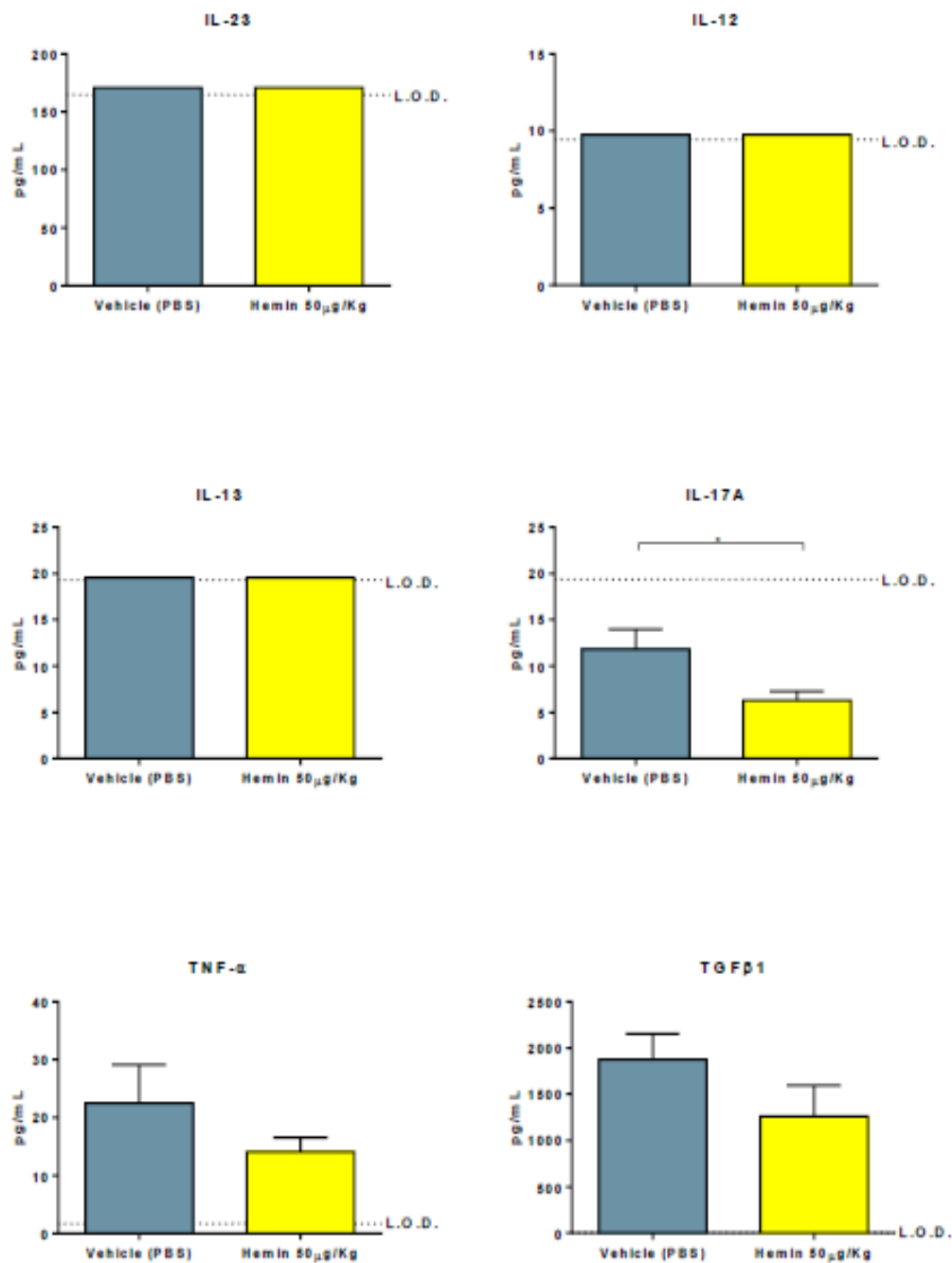


Figure 30. Day 35 Plasma Multiplex Graph Part 2. Plasma was collected at the timepoints indicated in Table 1. Samples were analyzed using multiplex ELISA. Data is represented as group average \pm SEM. n=8 per group. LOD: Limit of detection. *: $p < 0.05$ as assessed by one tailed Student's T test.

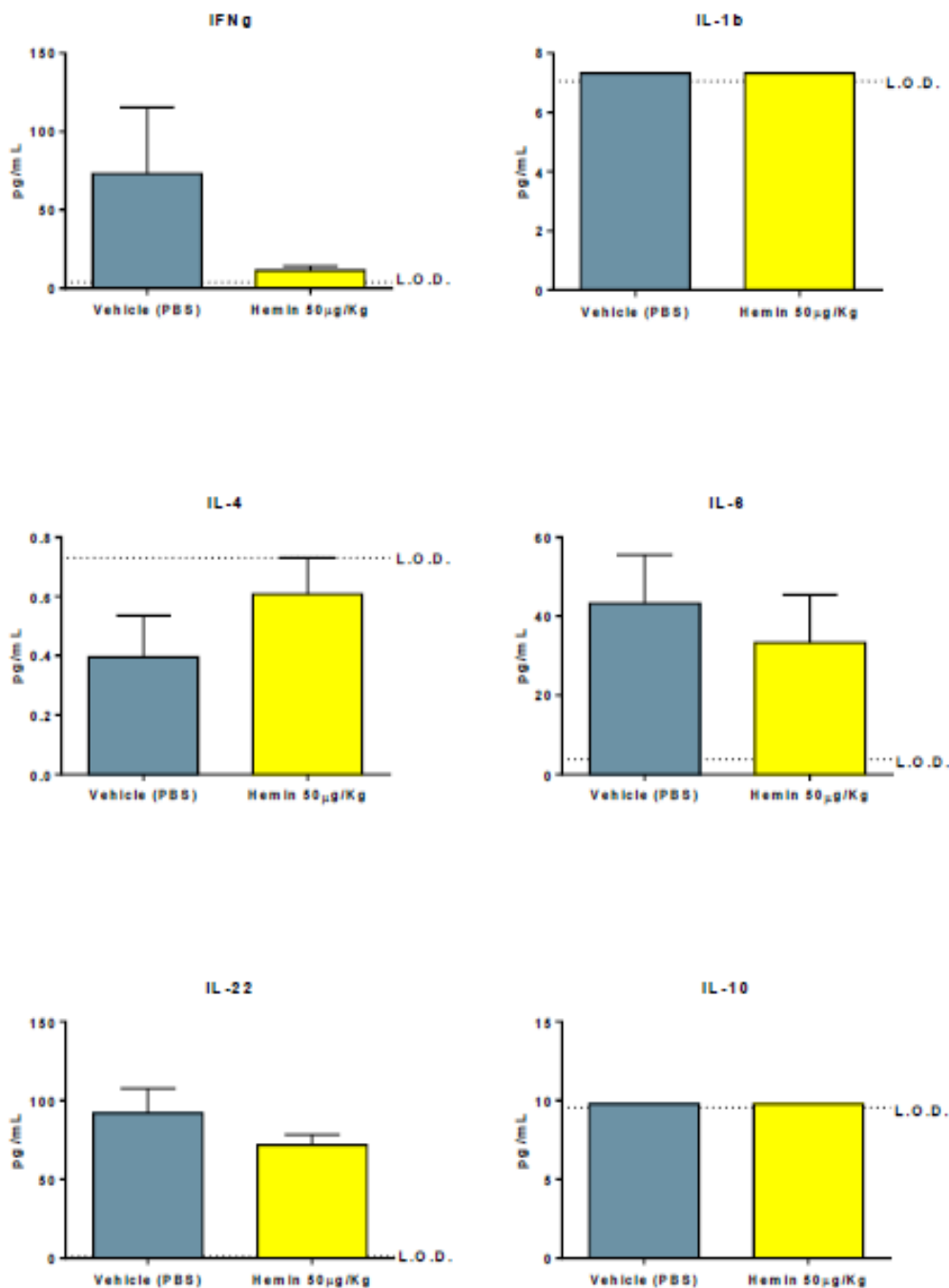


Figure 31. Day 49 Plasma Multiplex Graph Part 1. Plasma was collected at the timepoints indicated in Table 1. Samples were analyzed using multiplex ELISA. Data is represented as group average \pm SEM. n=8 per group. LOD: Limit of detection.

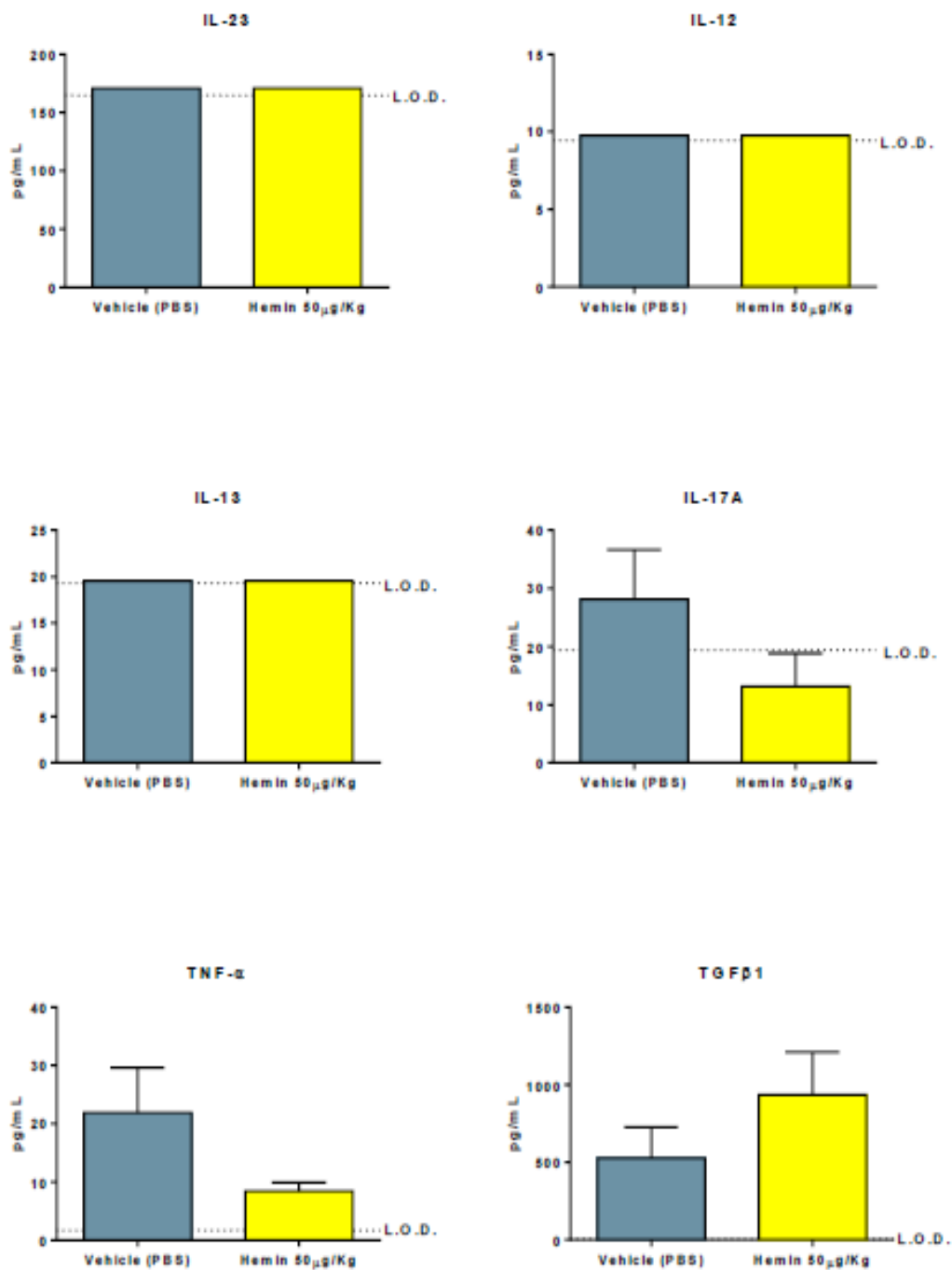


Figure 32. Day 49 Plasma Multiplex Graph Part 2. Plasma was collected at the timepoints indicated in Table 1. Samples were analyzed using multiplex ELISA. Data is represented as group average \pm SEM. n=8 per group. LOD: Limit of detection.

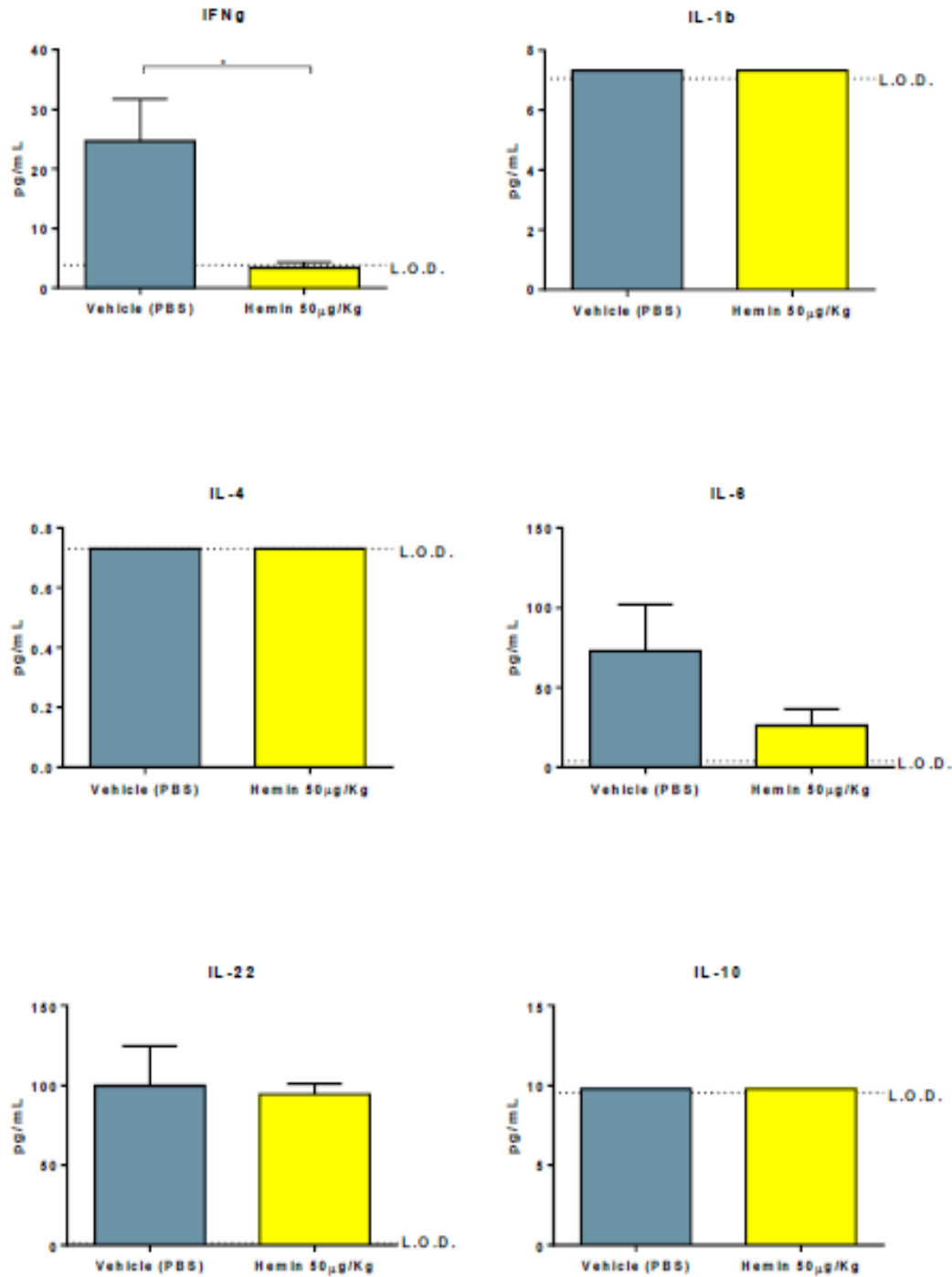


Figure 33. Day 56 Plasma Multiplex Graph Part 1. Plasma was collected at the timepoints indicated in Table 1. Samples were analyzed using multiplex ELISA. Data is represented as group average \pm SEM. n=8 per group. LOD: Limit of detection. *: $p < 0.05$ as assessed by one tailed Student's T test.

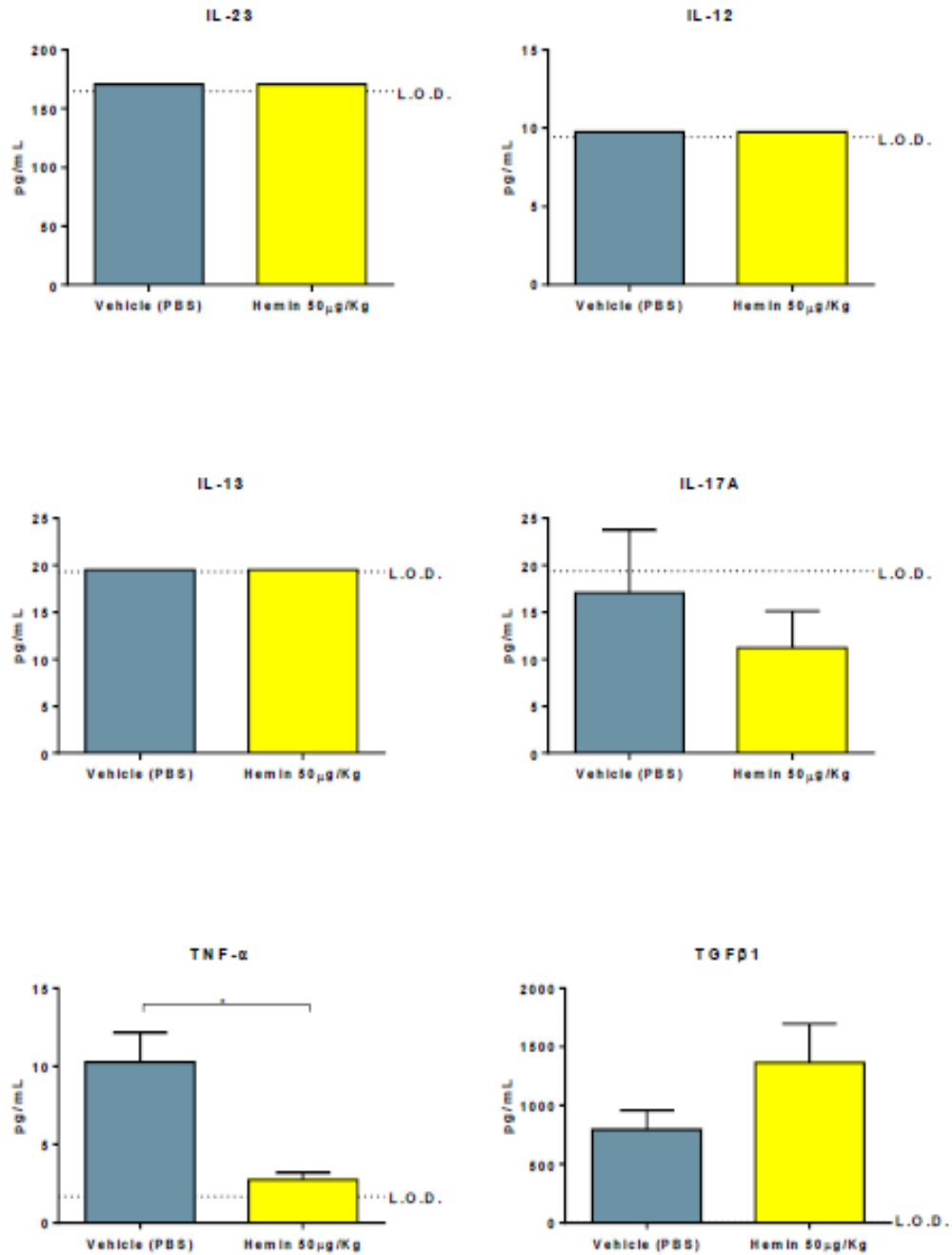


Figure 34. Day 56 Plasma Multiplex Graph Part 2. Plasma was collected at the timepoints indicated in Table 1. Samples were analyzed using multiplex ELISA. Data is represented as group average \pm SEM. n=8 per group. LOD: Limit of detection. *: $p < 0.05$ as assessed by one tailed Student's T test.

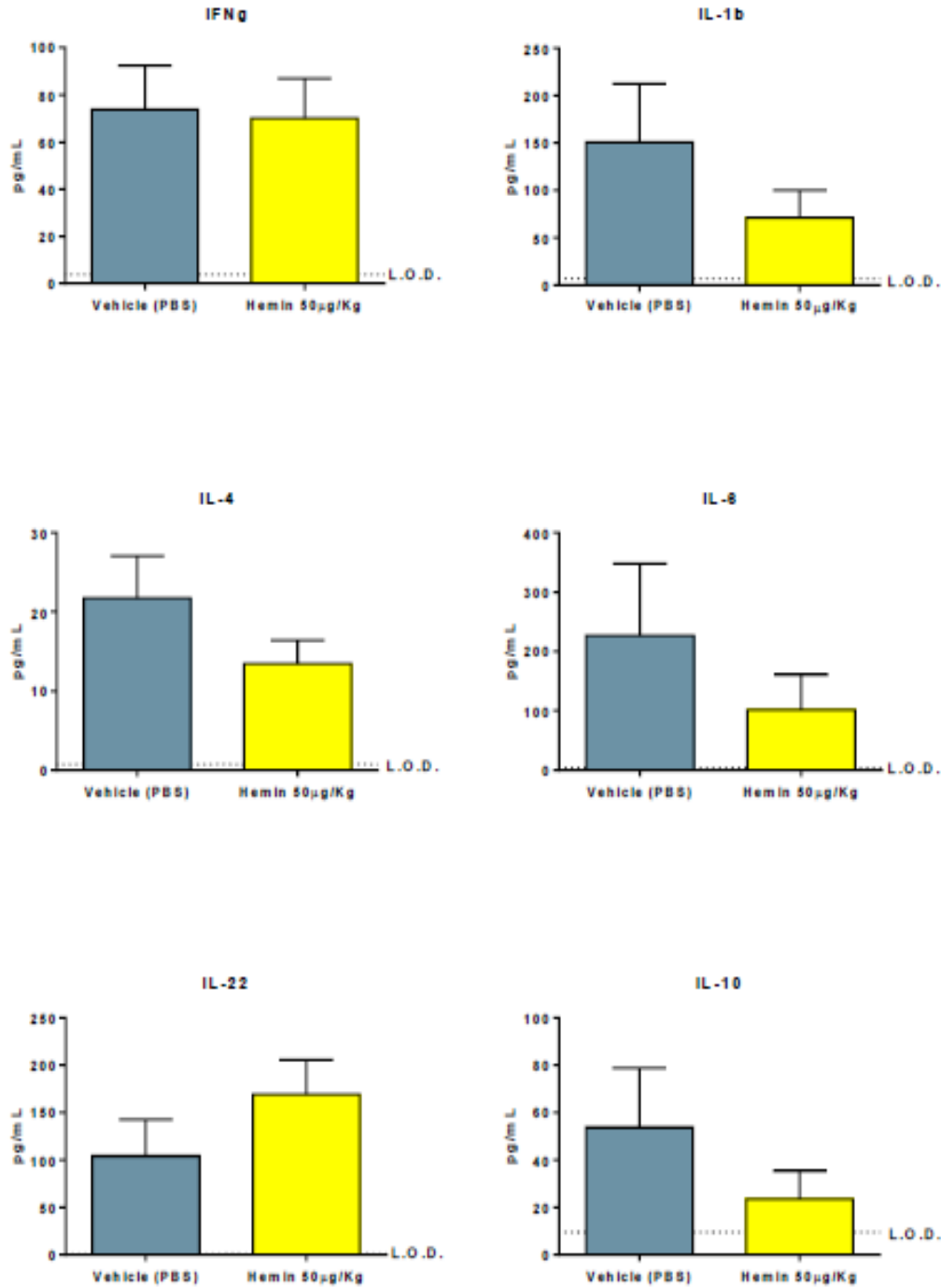


Figure 35. Day 56 Mesenteric Lymph Node Multiplex Graph Part 1. Plasma was collected at the timepoints indicated in Table 1. Samples were analyzed using multiplex ELISA. Data is represented as group average \pm SEM. n=8 per group. LOD: Limit of detection.

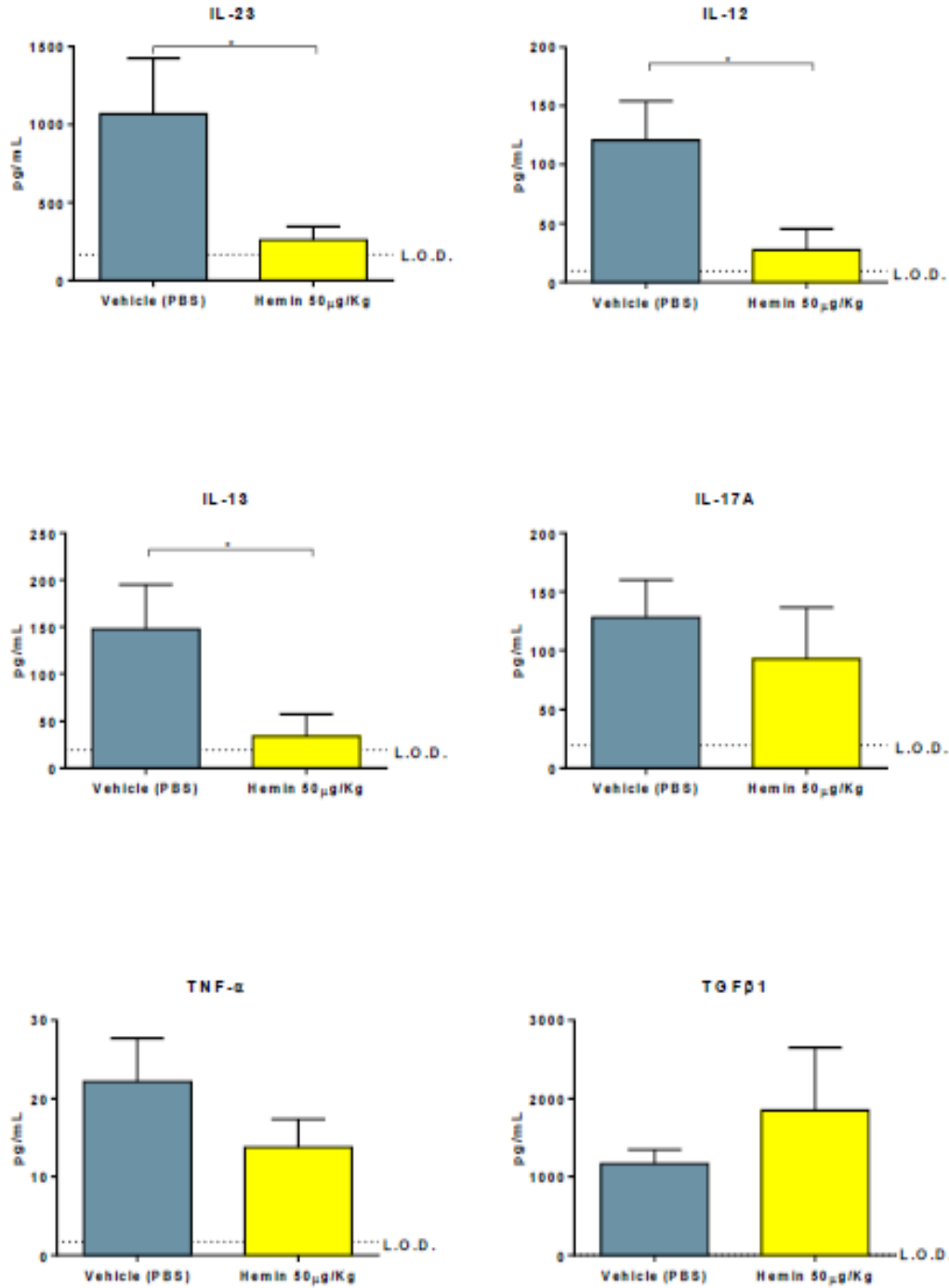


Figure 36. Day 56 Mesenteric Lymph Node Multiplex Graph Part 2. Plasma was collected at the timepoints indicated in Table 1. Samples were analyzed using multiplex ELISA. Data is represented as group average \pm SEM. n=8 per group. LOD: Limit of detection. *: $p < 0.05$ as assessed by one tailed Student's T test.

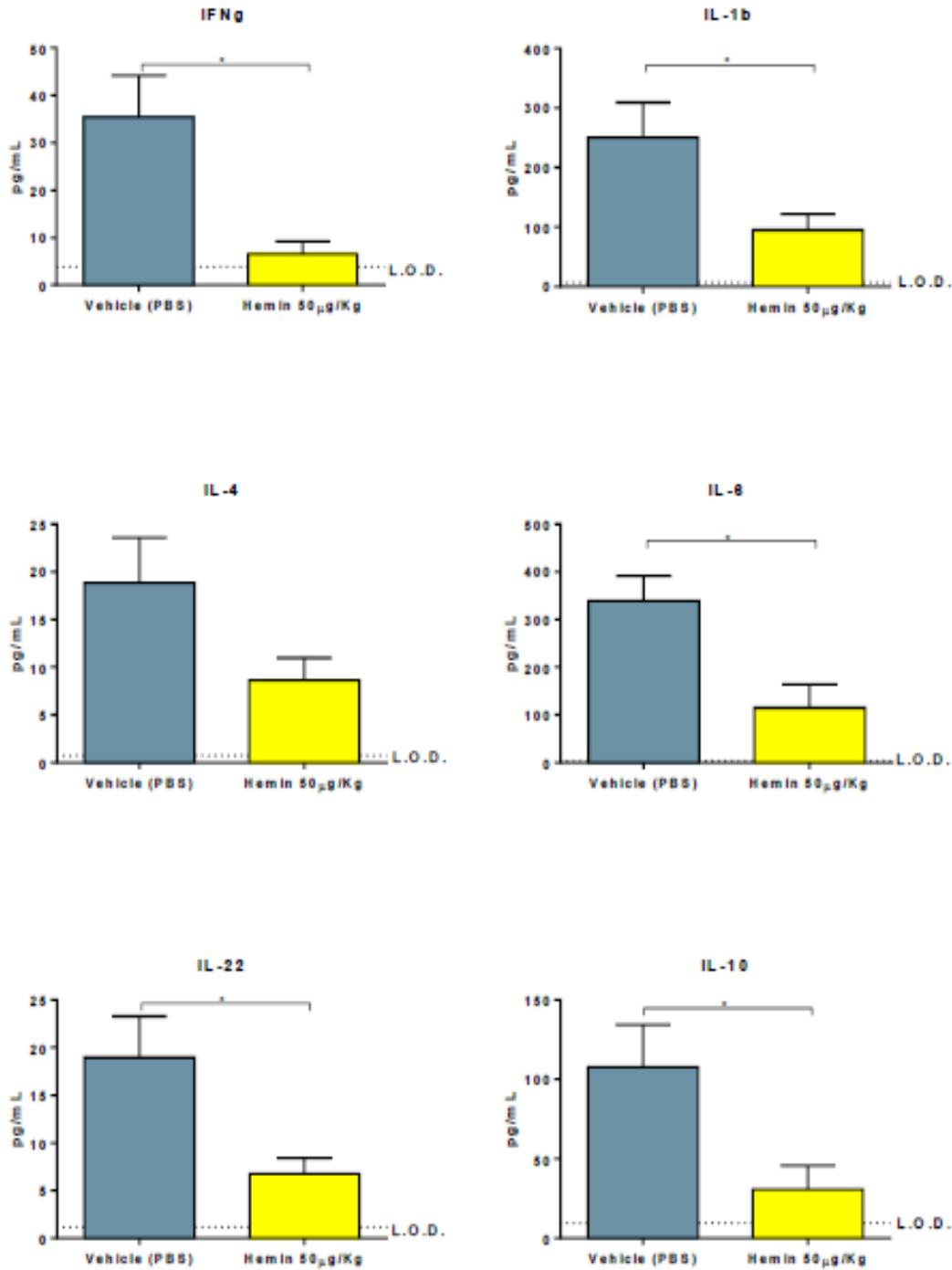


Figure 37. Day 56 Spleen Multiplex Graph Part 1. Plasma was collected at the timepoints indicated in Table 1. Samples were analyzed using multiplex ELISA. Data is represented as group average \pm SEM. n=8 per group. LOD: Limit of detection. *: p<0.05 as assessed by one tailed Student's T test.

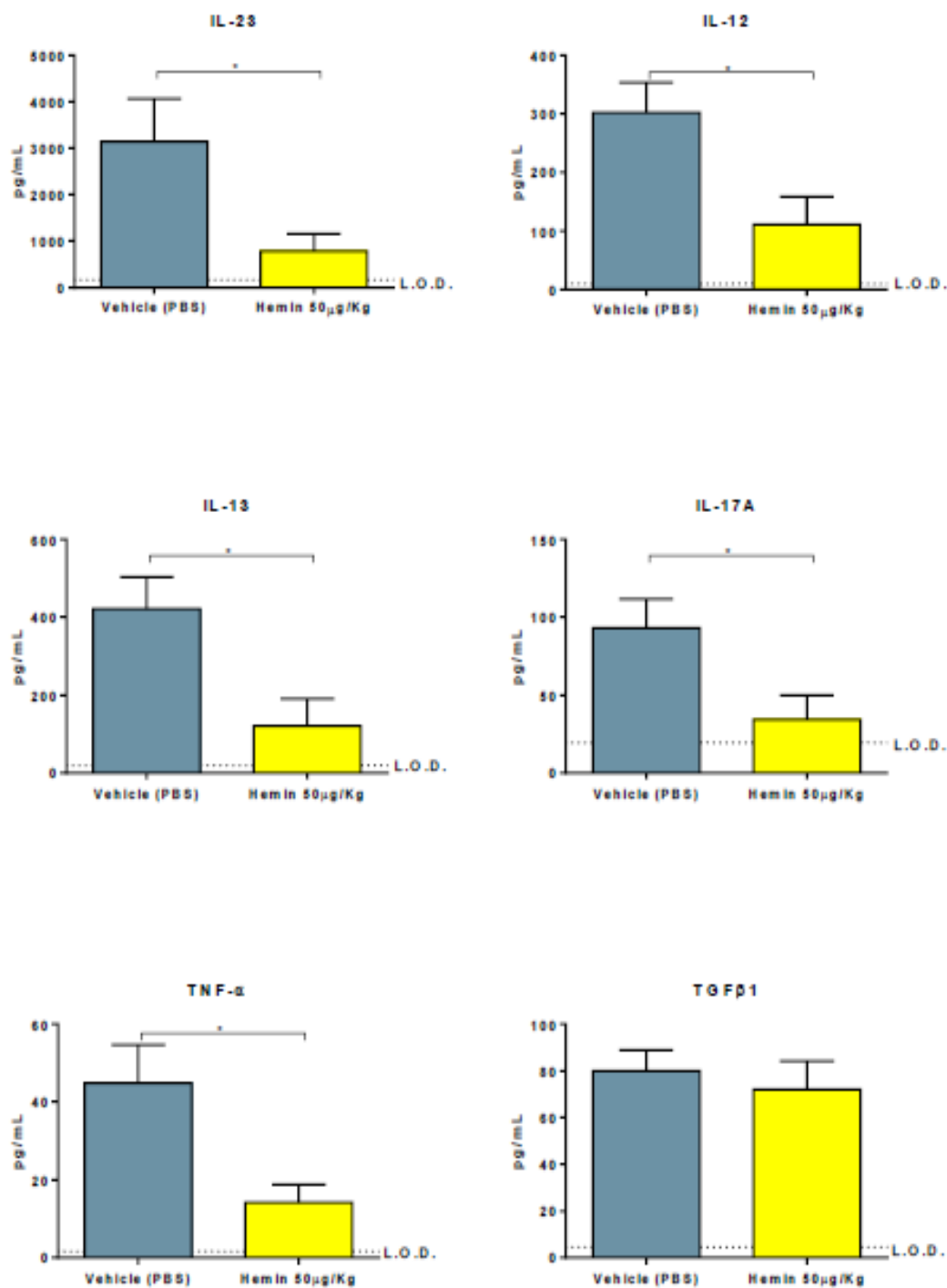


Figure 38. Day 56 Spleen Multiplex Graph Part 2. Plasma was collected at the timepoints indicated in Table 1. Samples were analyzed using multiplex ELISA. Data is represented as group average \pm SEM. n=8 per group. LOD: Limit of detection. *: $p < 0.05$ as assessed by one tailed Student's T test.

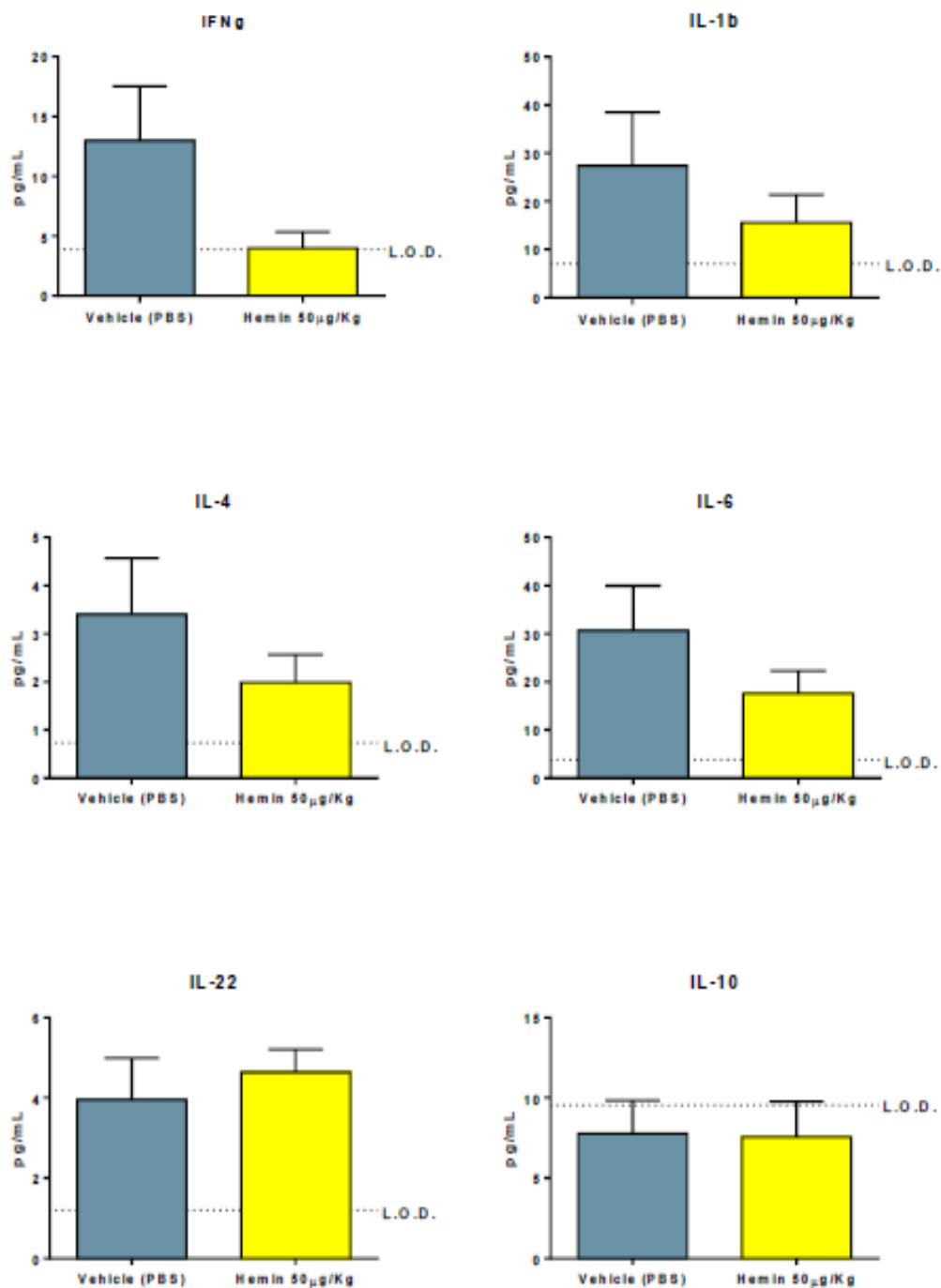


Figure 39. Day 56 Colon Multiplex Graph Part 1. Plasma was collected at the timepoints indicated in Table 1. Samples were analyzed using multiplex ELISA. Data is represented as group average \pm SEM. n=8 per group. LOD: Limit of detection.

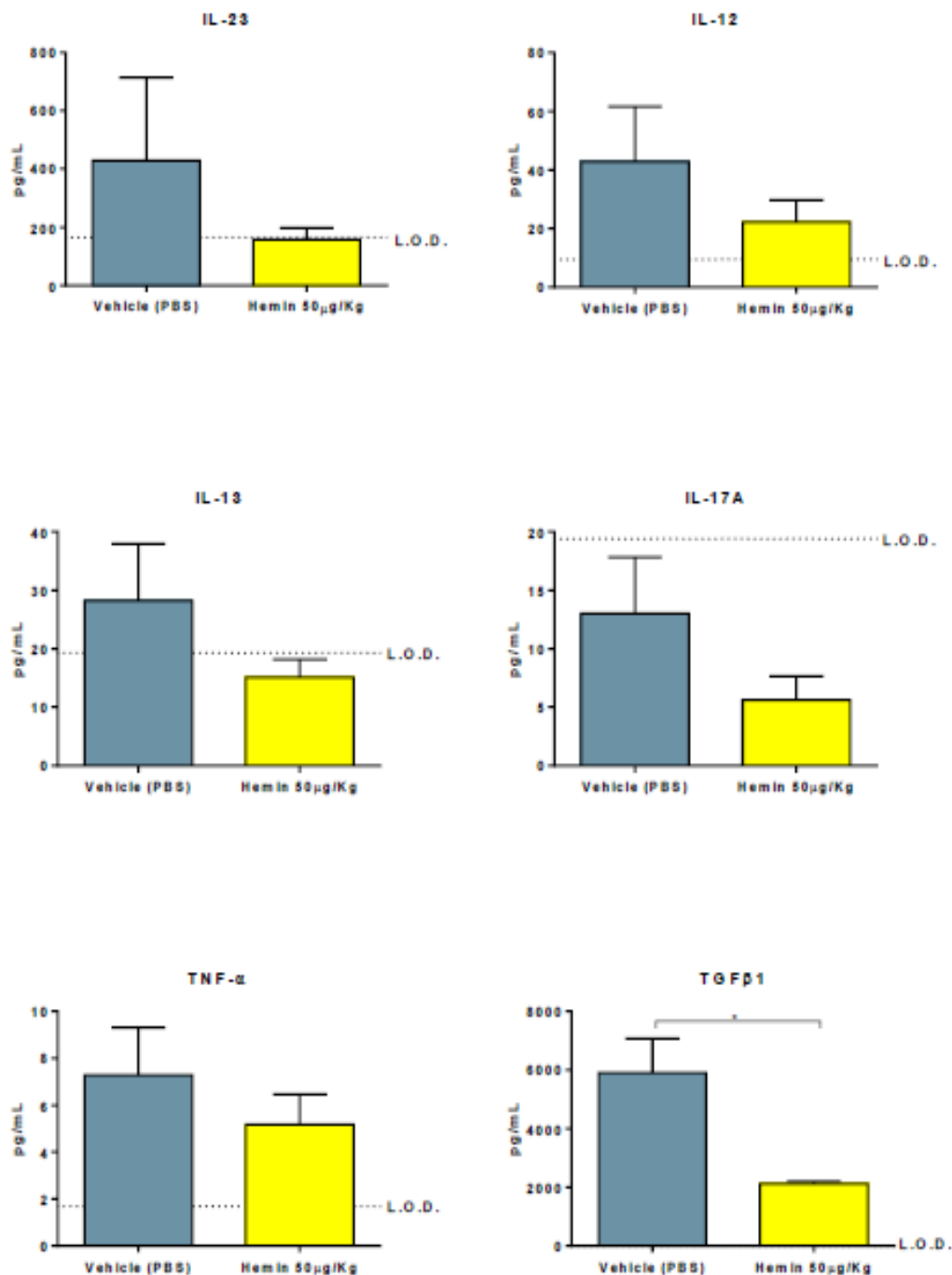


Figure 40. Day 56 Colon Multiplex Graph Part 2. Plasma was collected at the timepoints indicated in Table 1. Samples were analyzed using multiplex ELISA. Data is represented as group average \pm SEM. n=8 per group. LOD: Limit of detection. *: $p<0.05$ as assessed by one tailed Student's T test.

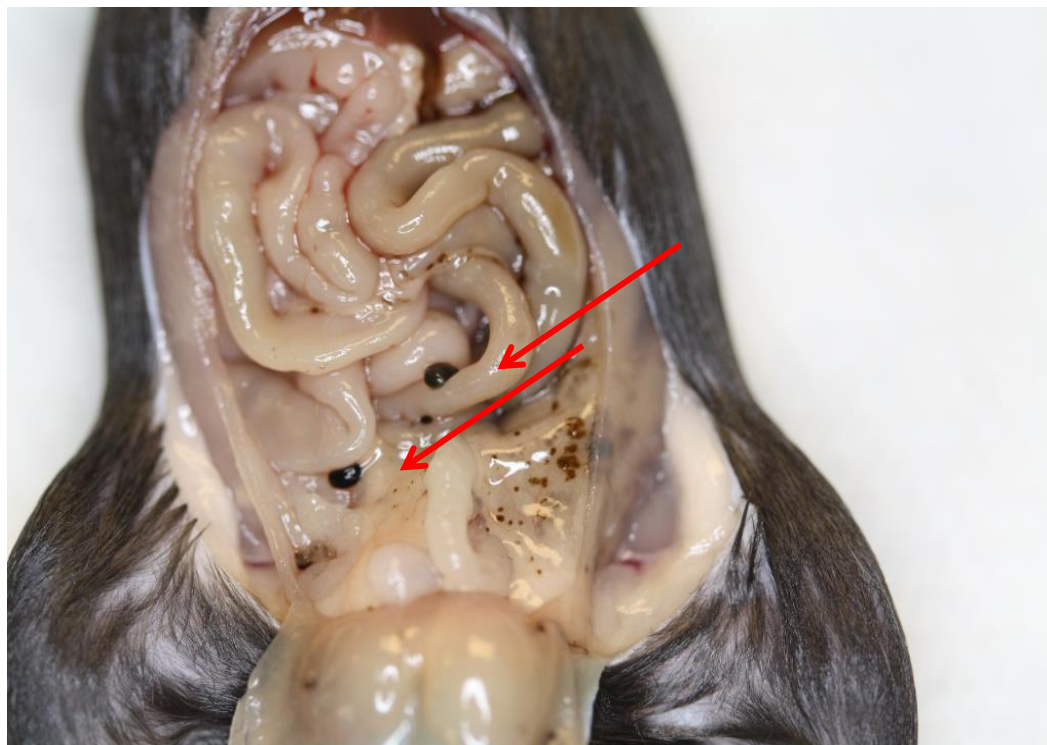


Figure 41. Representative Necropsy Photos. Group 1 (top) is normal and Group 2 (bottom) hemin deposits (red arrows) are seen in peritoneal cavity.

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